Common pathophysiologic pathways of diabetes mellitus and depression: focusing on the *brain-derived neurotrophic factor*

Ph.D. Thesis

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Introduction

Today diabetes mellitus (DM) is a major health concern which poses a huge social and economical burden on Euro-Atlantic societies. Depression is also a worldwide endemic, affecting millions. Joint occurrence of the diseases worsens quality of life and decreases life expectancy. Common pathophysiologic background of DM and depression has been proposed, but the exact pathomechanism in not known to-date.

Current research aims to clarify the relation of the diseases on a molecular level. Common immunological processes, similar reaction to oxidative stress as well as common endocrinologic and neurobiologic factors are suspected to be involved

Based on specific structural abnormalities the role of neurotrophins, especially brain-derived neurotrophic factor (BDNF) have emerged in the development of comorbidity. BDNF is predominantly produced in the central and peripheral nervous system, but can also be detected in non-neurogenic tissues as well.

Similarly to all neurotrophins BDNF is synthetized in the endoplasmic reticulum (ER) in a precursor form, from which the mature form is produced via proteolytic cleavage. Both the precursor and mature form are biologically active, however they have opposing functions. The precursor form binds to neurotrophin receptor p75 (p75Ntr), activates apoptotic pathways and decreases synaptic activity. On the other hand, mature BDNF induces cell survival, axon growth and synaptic activity through tropomyosin receptor kinase B (TrkB).

Animal and human studies have confirmed the central role of BDNF in the development of neuropsychiatric diseases. Under physiologic conditions neurons in the hippocampus and prefrontal region have high quantities of BDNF and TrkB, which decrease during depression. TrkB-mediated pathways are pivotal in the mode of action of antidepressants and agonism of TrkB has an antidepressant effect by promoting cell proliferation in the nervous system.

Sigma-1 receptor (S1R) is an ER-resident transmembrane chaperone, which is an important upstream regulator of BDNF. S1R agonism or overexpression increases chaperone activity and promotes precursor-mature BDNF transformation and mature BDNF secretion.

S1R has a number of endogenous (neurosteroids eg. Dehidroepiandrosterone) and pharmacologically relevant exogenous (neuroleptins, antidepressants, etc.) ligands, all of which either activate or antagonize the receptor. Many selective serotonin reuptake inhibitors – eg. Fluvoxamine (FLU) or sertaline – have high affinities for S1R and thus it is suspected that S1R partakes in the pharmacologic action of these antidepressants.

Depression in diabetic patients not only worsens disturbed carbohydrate metabolism, but also increases the frequency of complications. Of the many diseases developing as a consequence of DM, our group focuses on the pathomechanism of diabetic nephropathy (DNP) and possible new treatment options. During DNP systemic and renal renin-angiotensin-aldosterone system (RAAS) is activated and according to domestic and international guidelines RAAS-inhibitors are the primary option to slow the progression of kidney disease.

It has recently been proven that – partly independently from the periphery system – there is a local RAAS in the brain expressing all elements of the classic RAAS. Beside regulating blood pressure, RAAS in the brain also regulates body temperature and locomotor activity as well as memory, behavior and learning.

Angiotensin II (ANGII) functions as a stress hormone in the brain. It is produced in the brain, but can also pass through the blood-brain barrier from systemic circulation. ANG II is increased during acute or chronic stress, it induces inflammatory processes and activates the HPA-axis, which could lead to depressive-like behavior. Both animal and human studies confirm that increased local RAAS activity in tha brain is associated with diseases of the nervous system, such as Alzheimer's or Parkinson's disease or depression.

Recently new light has been thrown upon the pleiotropic effects of RAAS-inhibitors (anti-hypertensive, nephroprotective). It has been shown that in hypertensive patients taking ARBs or ACE-inhibitors depression is decreased, cognitive function is improved and less antidepressant drugs are needed than hypertensive patients taking beta-blockers. These clinical studies implied that RAAs-inhibitors have antidepressant properties.

During my Ph.D. work I aimed to investigate if S1R-BDNF signaling is involved in the development of depression during diabetes. I also studied the effect of RAAS-blockers - the primary treatment option for DNP – on depression and the pathomechanism behind their antidepressant effect.

Aims

1. Does S1R agonist FLU prevent DM-associated depression?

2. What is the effect of FLU on S1R – BDNF signaling in the brain?

3. Are RAAS-blockers effective in preventing DM-associated depression?

4. Which molecular mechanisms are responsible for the protective effect of RAAS inhibitors?

Methods

Type 1 diabetes mellitus (T1DM) model and treatment protocol

Adult, male Wistar rats weighing 200±15g were used in all experiments. T1DM was induced by a single intraperitoneal (*ip.*) injection of 65 mg/bwkg streptozotocin. After five weeks of T1DM rats were randomly divided into groups (n=6-8/group) and were treated for two weeks by oral gavage as follows:

I. Experiment: Antidepressant FLU treatment:

1. **Control:** isotonic saline as vehicle
2. **Diabetic (D)**: diabetic animals, isotonic saline as vehicle
3. **D+FLU20**: diabetic animals, 20 mg/bwkg/day **fluvoxamin-maleat** (**FLU**) dissolved in saline
4. **D+FLU2**: diabetic animals, 2 mg/bwkg/day **fluvoxamin-maleat** (**FLU**) dissolved in saline
5. **D+FLU20+NE100**: diabetic animals 20 mg/bwkg/day **FLU** + 1 mg/bwkg/day **specific S1R antagonist** N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (**NE100**)
6. **D+FLU2+NE100**: diabetic animals, 2 mg/bwkg/day **FLU** + 1 mg/bwkg/day **NE100**

II. Experiment: RAAS inhibitors treatment:

1. **Control:** isotonic saline as vehicle
2. **Diabetic (D)**: diabetic animals, isotonic saline as vehicle
3. **D+ENA**: diabetic animals, 40 mg/bwkg/day angiotensin-converting-enzyme (ACE) inhibitor **enalapril** dissolved in saline
4. **D+RAM**: diabetic animals, 10 μg/bwkg/day angiotensin-converting-enzyme (ACE) inhibitor **ramipril** dissolved in saline

5. **D+LOZ**: diabetic animals, 20 mg/bwkg/day angiotensin-receptor blocker (ARB) **losartan** dissolved in saline
6. **D+SPI**: diabetic animals, 50 mg/bwkg/day non-selective aldosterone antagonist **spironolacton** dissolved in saline
7. **D+EPL**: diabetic animals, 50 mg/bwkg/day selective aldosterone antagonist **eplerenone** dissolved in saline

Age-matched, non-diabetic control rats (C) were treated with saline by oral gavage daily for two weeks at the same time as the diabetic animals.

Before the harvest 24 h urine was collected. Blood samples were taken from the abdominal aorta. Brain, thymus, adrenal glands and kidney were collected; hippocampus and prefrontal area were separated.

Experiments

Behaviour tests

Three days before the end of the 7-week experimental period all animals were tested for locomotor activity by open field test. Depressive-like behaviour was evaluated by forced swim test; the pre-test was measured 24 hours after the open field test and the test session was conducted 24 hours after the pre-test.

Open field test

The open field test was performed in a square arena surrounded by a wall (100 x 100 x 60 cm box). The floor was virtually divided into squares (10 x 10 cm). Rats were individually placed into the centre of the field and allowed to explore freely for 10 min. Horizontal locomotory parameter (number of squares crossed) was evaluated manually.

Forced swim test

Rats were placed in the cylinder (60 cm tall, 14 cm in diameter filled with tap water (24 ± 1°C) at a height of 30 cm). The animals were forced to swim for a 15 min period (pre-test) and 24 h later were subjected to 5 min test session. Time of mobility parameters (swimming, diving, struggling) and immobility parameters (floating) were analysed.

Blood pressure

Systolic, diastolic blood pressure and pulse were measured by CODA standard tail-cuff monitoring system before and after the 2 weeks RAAS-treatment period.

Renal functional and metabolic parameters

The following serum laboratory parameters were measured: glucose, fructosamine, creatinine, urea nitrogen, potassium, triglycerides, cholesterol, glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT). Creatinine, urea nitrogen and glucose were determined from the 24 h urine.

Histology

Mesangial matrix expansion was evaluated on periodic acid-Schiff stained kidney sections. Fibronectin immune staining was used to evaluate tubulointerstitial fibrosis.

Enzyme-linked immunosorbent assay (ELISA)

Serum BDNF and urinary albumin concentration was measured by sandwich enzyme-linked immunosorbent assay.

Measurement of mRNA expression

Brain *Bdnf*, *Sigmar1*, *Bcl2* és *Bax* mRNA expression were determined using quantitative real-time PCR and normalized against *Rn18s* as a housekeeping gene.

Protein abundance measurements

Western blot was used to measure the following proteins: BDNF, p75Ntr, phospho-ERK1/2, phospho-CREB, S1R, TrkB. Bands of interest were factored for Ponceau red staining to correct for any variations in total protein loading

Statistical analysis

Statistical analyses were performed using Prism software (version 6.00). Multiple comparisons and possible interactions were evaluated by one-way ANOVA followed by Bonferroni post-hoc test. For non-parametrical data the Kruskal–Wallis ANOVA on ranks followed by Dunns post-hoc test. Data are expressed as means ± SEM. *P* values of <0.05 were considered significant.

Results

Effect of FLU on metabolic parameters

Blood glucose and fructosamine levels were elevated in the DM group seven weeks after the STZ injection. Increased cholesterol and triglyceride levels confirmed disturbed lipid metabolism caused by diabetes. Weight gain of diabetic rats was less than that of the control group. FLU treatment decreased serum lipid levels in both dosages, but did not affect any of the other measured parameters.

Neuroendocrine parameters and serum BDNF levels

Both depression and diabetes are characterized by distinct organ alterations (decreased thymus weight, hypertophic adrenal glands). In our experiments we detected hypetrophic adrenal glands and decreased thymus weights in the DM group. FLU treatment had no effect on these parameters.

Chronic stress such as depression or diabetes is known to alter serum BDNF. We confirmed decreased serum BDNF levels in DM, which was not affected by any of the treatments.

FLU mitigates DM-induced depressive-like behavior

Depression developed after 7 weeks of DM, which was characterized by increased floating time

Antidepressant FLU treatment dose-dependently – only the 20 mg/bwkg dosage – improved depressive-like behavior. Specific S1R antagonist NE100 suspended this beneficial effect of FLU.

The effect of various treatments on locomotor activity was assessed using the open-field test. Antidepressant treatment did not improve physical activity. This confirms that higher dose FLU treatment had an antidepressant effect without affecting the physical condition of the animals.

FLU increases BDNF and S1R protein levels

BDNF and S1R protein levels were measured in the hippocampus and prefrontal area, as these brain regions are the most affected during depression and diabetes. 2 mg/bwkg FLU dosage did not improve depressive-like behavior, thus protein levels were only measured after 20 mg/bwkg treatment.

Precursor and mature BDNF as well as S1R protein decreased in the hippocampus and prefrontal area of diabetic rats. Both proteins were increased by FLU and this was blocked by the addition of S1R antagonist.

DM and FLU do not affect Bdnf and Sigmar1 mRNA expression

Neither DM nor the treatments had any effect on Bdnf and Sigmar1 mRNA expression in the investigated brain regions.

Confirmation of non-pressor RAAS-inhibitor dosages

RAAS inhibitor dosages were determined based on our earlier experiments and literary data in order to inhibit RAAS expression and activity without altering blood pressure. Blood pressure of diabetic rats remained unaltered in all groups throughout the experiments.

Effect of RAAS-inhibitors on metabolic parameters

In the II. series of experiments the metabolic alterations were similar to the I. series in the diabetic group at the end of the 7 week period: body weight decreased, serum glucose, fructosamine and lipid levels increased. Enalapril and eplerenone prevented weight loss. Enalapril and aldosterone antagonists effectively improved lipid metabolism.

RAAS-inhibitors improve kidney function

Functional deterioration in diabetic rats was characteristic to DNP: kidney/body weight ratio increased, serum creatinine and urea nitrogen levels increased, glucose clearence and microalbuminuria were elevated and creatinine clearence was decreased.

RAAS-inhibitors decreased kidney hypertrophy and glucose clearence, while they also improved urea nitrogen levels and creatinine clearence.

RAAS-blockers ameliorate structural and fibrotic deterioration in DNP

Mesangial matrix expansion was evaluated on PAS-stained kidney sections. Capillary lumen areas were decreased and mesangial matrix expansion was extensive in DM. All RAAS-blockers mitigated DM-induced mesangial matrix expansion and subsequent glomerular damage.

During fibrosis fibronectin, the main component of extracellular matrix is produced in excessive amounts. Massive amounts of fibronectin was detected in glomeruli and to a lesser extent in the tubulointerstitial region of diabetic rats. RAAS-inhibitors effectively hindered fibronectin production

Alterations of neuroendocrine parameters and serum BDNF levels

Neuroendocrine parameters of the DM group were similar in the II. series of experiments to the I. series: adrenal gland weight increased and thymus weight decreased compared to control animals. RAAS-blockers had no effect on thymus weight. ACE-inhibitor enalapril, ramipril and ARB losartan had no effect, while aldosterone antagonists eplerenone and spironolactone increased adrenal gland weight.

Similarly to the I. series of experiments serum BDNF decreased in diabetic rats and RAAS inhibitors had no effect.

RAAS-blockers improve DM-induced depressive-like behaviour

All RAAS-inhibitors increased mobility time, which proved their antidepressant properties. Similarly, specific mobility parameters showed an improving tendency when measured separately in all RAAS-inhibitor treated rats. ACE-inhibitor enalapril and ramipril significantly improved the swimming parameter. RAAS-blockers did not improve the physical condition of the rats.

Effect of RAAS-blockers on precursor and mature BDNF in the brain of T1DM rats

Both precursor and mature BDNF levels decreased in the hippocampus of diabetic rats. RAAS-blockers not only suppressed the decline of BDNF levels, but caused a compensatory increase (except aldosterone antagonists).

RAAS-blockers enhance TrkB - ERK - CREB - Bcl2 signaling in the T1DM rat brain

In our experiments TrkB – the receptor of mature BDNF – was decreased in DM. In parallel, proteins of the signaling pathway activated by TrkB, such as ERK1/2 and phospho-CREB were also deceased in diabetic rats.

RAAS-inhibitors – except for ramipril – increased both expression of the receptor and phospho-ERK1/2 and phospho-CREB levels. Anti-apoptotic *Bcl2* mRNA expression was decreased in the diabetic hippocampus, but was increased by RAAS-inhibitors.

RAAS-inhibitors do not affect p75Ntr - JNK - Bax signaling in the T1DM brain

Main members of precursor BDNF signaling include p75Ntr - JNK – Bax. Neither DM, nor RAAS-inhibitors had any effect on p75Ntr and phospho-JNK levels in the hippocampus. In parallel, pro-apoptotic *Bax* mRNA expression remained unchanged in all groups as well. According to our results downstream signaling of precursor BDNF is not activated in this experimental setup.

Conclusions

1. We confirmed that antidepressant FLU treatment dose-dependently mitigates T1DM-associated depression.

2. We showed that S1R-BDNF signaling is involved in the pathomechanism of T1DM-associated depression. In T1DM S1R agonist treatment increases BDNF levels in the brain regions involved in the development of depression: the hippocampus and the prefrontal cortex.

3. We were the first to show that not only ARBs, but long-term, non-pressor dose ACE-inhibitor and aldosterone antagonist treatment also has an antidepressant effect in T1DM.

4. The BDNF - TrkB - CREB signaling pathway is crucial in the pathomechanism of T1DM-associated depression and takes part in the antidepressant effect of RAAS-inhibitors by promoting neuron survival.

5. Pro-apoptotic signaling induced by precursor BDNF - p75Ntr, is not involved in the development of T1DM-associated depression or the antidepressant effect of RAAS-blockers.

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