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Complete List of Authors:	Pechlivanova, Daniela; Institute of Neurobiology, Bulgarian Academy of Sciences, Petrov, Kiril; Medical University-Sofia, Department of Pathophysiology Grozdanov, Petar; Institute of Microbiology, Bulgarian Academy of Sciences Nenchovska, Zlatina; Institute of Neurobiology, Bulgarian Academy of Sciences Tchekalarova, Jana; Institute of Neurobiology, Bulgarian Academy of Sciences Stoynev, Alexander; Medical University-Sofia, Department of Pathophysiology
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Intracerebroventricular infusion of an Angiotensin AT2 receptor agonist Novokinin aggravates some diabetes mellitus-induced alterations in Wistar rats

Pechlivanova D^{1*}, Petrov K.², Grozdanov P.³, Nenchovska Z.¹, Tchekalarova J.¹, Stoynev A.²

¹*Institute of Neurobiology, Bulgarian Academy of Sciences.*

²*Department of Pathophysiology, Medical University-Sofia*

³*Institute of Microbiology, Bulgarian Academy of Sciences.*

* *Corresponding author:* Pechlivanova, Daniela M., Institute of Neurobiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl.23, Sofia 1113, Bulgaria

Tel.: 359-2-9792151

Fax: 359-2-8719109

e-mail: pechlivanova@yahoo.com

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Abstract

Cumulative data suggest the significant role of the Renin - Angiotensin System in the development of the pathological consequences of diabetes mellitus (DM). Newly synthesized AT₂ receptor agonists gained importance as a target for creating new antihypertensives. The aim of the present work was to study the effects of a peptide AT₂ agonist Novokinin, infused intracerebroventricularly, on the consequences of the streptozotocin-induced type 1 DM (T1 DM) in Wistar rats. Food and water consumption, body weight, urine excretion (metabolic cages), motor activity (open field test), anxiety (elevated plus maze), nociception (paw pressure analgesimeter test), spatial memory (T-maze alternation test) and plasma levels of glucose and corticosterone (ELISA) were assessed two weeks after the T1 DM induction.

Novokinin increased water and food consumption, and urine output and reduced weight gain in the control rats. Diabetic rats demonstrated hyperalgesia, increased level of plasma corticosterone, decreased motor and exploratory activity, impaired spatial memory. Novokinin infusion increased water intake, diuresis and mortality rate, decreased food intake, exacerbated diabetes-induced hyperalgesia and provoked anxiety-like behavior but improved spatial memory in diabetic rats.

These initial data suggest that angiotensin AT₂ receptors participate in the pathogenesis of T1 DM-induced complications in the function of the nervous system.

Key words: Angiotensin AT₂ receptors; Diabetes mellitus; Behavior; Corticosterone; Rats

Introduction

Diabetes mellitus (DM) is accompanied by chronic complications, such as neuropathic pain, loss of sensation and impaired motor coordination (Feldman et al. 2017; Timar et al. 2016). In some cases DM might be a prerequisite for brain atrophy, abnormalities in the white and gray matter of the brain, learning disability and increased risk of dementia (Gispen and Biessels 2000).

Experimental evidence indicates that renin-angiotensin system (RAS) play an important role in the development of pathological consequences of DM (Wolf and Ziyadeh 1997). The main biologically active peptide of RAS - angiotensin II (Ang II) binds to specific AT1 and AT2 receptors that have uneven distribution into the tissues. AT1 receptors are involved mainly in the regulation of arterial blood pressure, water-salt balance, behaviors associated with drinking water and more complex activities controlled by central nervous system (De Gasparo et al. 2000).

Relatively less studied AT2 receptors are involved in sensory-motor functions, neuro-degenerative processes, thermoregulation; in some physiological functions AT2 receptors act as AT1 receptors antagonist, while in others both receptors act synergistically (Danyel et al. 2013). Experimental and clinical data show that angiotensin converting enzyme (ACE) inhibitors and AT1 receptor blockers improve insulin resistance, decrease proteinuria and renal hypertrophy in humans with DM (Lewis et al. 2001; Thurman and Schrier 2003) and in streptozotocin diabetic model in rats (Erman et al. 2004). Data suggest that their beneficial effects are not only the result of blocked AT1 receptors, but also of an activation of the AT2 receptors by the endogenous Ang II (Naito et al. 2010). The density of AT2 receptors is increased in pathological conditions such as heart and kidney failure, skin lesions, vascular damage, myocardial infarction, cerebral ischemia, and peripheral nerve damage (De Gasparo et al. 2000) and they may act as a functional opponent of AT1 receptors in the regulation of blood pressure and inflammatory responses (Yamada et al. 2009; Unger and Dablof 2010). On the other hand it was found a significant reduction in the glomerular AT2 receptors of rats during the early stages of diabetes whereas mRNA for renin, angiotensinogen, ACE or AT1 receptors were not changed (Wehbi et al. 2001).

Synthesis of selective AT2 receptor agonists as potential antihypertensive agents is a relatively new pharmacological target (Brouwers et al. 2015; Carey 2016). Our previous data demonstrated that AT2 receptors participate in the modulation of visceral pain, circadian rhythms of acute pain sensitivity, and participate in the regulation of the intake of water and food as well as excretion of urine (Georgieva and Georgiev 1999; Pechlivanova et al. 2013; Pechlivanova and

Stoynev 2013). The peptide AT₂ agonist Novokinin, which is modified ovokinin derived from ovalbumin (Yamada et al. 2008a; Yamada et al. 2008b), possesses high affinity for AT₂ receptor and exerts an antihypertensive effect after oral administration in spontaneously hypertensive rats (Yamada et al. 2008b). Recently published data revealed an inhibitory effect of novokinin on gastric acid secretion that is probably mediated via the brain AT₂ receptors with participation of endogenous prostaglandins and the antioxidant systems (Zhang et al. 2016).

The antihypertensive drugs are a part of the complex therapy of the DM, therefore it is important to assess their effects on both the pathogenesis of diabetes and the regulation of the behavior. The objective of this study is to determine the impact of an intracerebral infusion with the AT₂ receptor agonist Novokinin on metabolic, nociceptive and behavioral abnormalities associated with the development of type 1 diabetes mellitus (T1DM) in male Wistar rats.

1. Materials and methods.

1.1. Subjects and drugs

Wistar male rats, 12 weeks old at the onset of study, were used (Breeding facility of Bulgarian Academy of Sciences). The animals were kept under standardized conditions: temperature $21 \pm 2^{\circ}\text{C}$, artificial photoperiod cycle 12h/12h (light on 08:00-20:00 h) with light intensity of about 250 lux at the level of the cages, in individual metabolic cages and fed with a regular rodent diet and tap water ad libitum.

Novokinin (L-arginyl-L-prolyl-L-leucyl-L-lysyl-L-prolyl-L-tryptophan, NVK, Sigma Aldrich, Sofia, Bulgaria) was dissolved in sterile saline and infused intracerebroventricularly (ICV), chronically at a dose of $0.6 \mu\text{g}/\text{rat}/\text{day}$ for 28 days by osmotic minipumps (Alzet, Cupertino, CA, USA, model 2004) which deliver at $0.25 \mu\text{L}/\text{h}$ connected with brain kits 2 (Alzet, Cupertino, CA, USA). The pumps were inserted s.c. under anesthesia (Ketalar, 100 mg/kg, i.m., Xylazine 5 mg/kg, i.p, Sigma Aldrich, Sofia, Bulgaria) between the scapulae in a small pocket formed using a haemostat. Brain kits for chronic infusion of the peptide were implanted in the right lateral brain ventricle with coordinates 2 mm lateral and 1.5 mm posterior to Bregma and 4 mm deep from skull surface (Paxinos and Watson 1998) and fixed on the skull through screws and dental cement. Control rats were implanted with saline filled pumps. The dose was consistent with literature data for the same acute dose (Ohinata et al. 2009). During the first 14 days of treatment with NVK at a dose of $0.6 \mu\text{g} / \text{rat}$, we monitored the animals periodically (once a day for 90 minutes) for signs of toxicity: tremors, convulsions, salivation, diarrhea, lethargy, and

coma, and we did not observe any abnormalities, except those reported in the manuscript. The correct placement of the brain kits was verified histologically after the end of the experiment and two rats with incorrect implantations were excluded from the statistics.

All behavioral studies were conducted between 10:00 am and 13:00 pm during the autumn. The experimental design is presented on Fig. 1. Every experimental group included 10 rats at the beginning of the experiments: WIS- healthy controls; WIS- with T1DM; WIS- with T1DM treated with Novokinin.

Experiments were approved by the local ethical committee of Institute of Neurobiology, Bulgarian Academy of Sciences in accordance with EC Directive 2010/63/EU for animal experiments and *Guide to the Care and Use of Experimental Animals* (Vol. 1, 2nd Ed., 1993, and Vol. 2, 1984, available from the Canadian Council on Animal Care (CCAC), 190 O'Connor St, Suite 800, Ottawa, ON K2P 2R3, Canada.

1.2. Experimental type1 diabetes mellitus

The experimental model of type1 diabetes mellitus (T1DM) induced by injection of streptozotocin (STZ) in rats, is widely used to study the mechanisms of the disease, and for screening of new drugs (Junod et al. 1969). STZ (Sigma Aldrich) was injected intraperitoneally into overnight fasted rats at a dose of 65mg/kg, freshly diluted in citrate buffer (pH = 4.5). DM was confirmed 48 hours later by elevated plasma glucose level above 16 mmol/l (Accu-Chek® test strips). The blood samples were obtained from the tail vein through short lasting (up to 1 minute) immobilization and prick of the blood vessel. Only hyperglycemic animals were included in the experiments. Food and water intake, body weight and urine output were monitored daily using metabolic cages.

1.3. Behavioral methods

1.3.1. Open field test

The effects of T1DM and/or drug treatment on the motor activity (trajectory length in particular zones) were estimated in open field test by the SMART video tracking system (Harvard Apparatus, US). The procedure is described in details elsewhere (Petrov et al. 2013) and consisted of placing an animal in the center of an open-field (100 X 100 X 60 cm) for 5 min. The box was divided into two zones, outer (peripheral) and inner (central- 60x60 cm) squares, the latter been considered an aversive place for the rats. For each rat, the length of peripheral and

central trajectory were recorded. After each trial the field was thoroughly cleaned with 0.1% acetic acid solution to avoid any odorous traces.

1.3.2. Elevated plus maze (EPM)

EPM is accepted a major test in the study of anxiety behavior (Pellow and File 1986) and comprised two open arms (50×10 cm), provided with a small rim (1.5 cm) to avoid falling down of rats, two enclosed arms (50×10×40 cm), and a central platform (10×10 cm). The apparatus was elevated 50 cm above the floor level. Each rat was placed on the central platform facing an open arm and observed for 5 min. Total trajectory traveled, the ratio open arms / total trajectory and time spent in open arms /total time were recorded by SMART video tracking system (Panlab, Harvard Apparatus) and calculated.

1.3.3. Paw-pressure test

The paw pressure withdrawal reflex was determined with an analgesimeter (Ugo Basile, Italy). The mechanical pressure (in grams) required eliciting pain responses such as withdrawal or struggle was established as a mechanical pain threshold. The mechanical nociceptive threshold testing was optimized by single training of the animals 1 day before the experiments (Anseloni et al. 2003).

1.3.4. T-maze rewarded alternation test

The T-maze rewarded alternation (TMRA) test is used to access working memory ability. Alternation reflects the motivation of the animal to explore its environment and locate the presence of resources such as food, water, mates or shelter (Deacon and Rawlins 2006). The apparatus (Columbus maze), made of stainless steel with removable transparent covers, consists of a start arm (42 cm long, 11.4cm wide and 11.4cm height) joined perpendicularly with two identical goal arms (42 cm long, 11.4cm wide and 11.4cm height) through a central area. Each arm is provided with a guillotine door at the central area and each goal arm is provided with a food well (3 cm in diameter and 1 cm deep) at its distal end. Before the training, rats were maintained at a restricted feeding schedule and habituation procedure. The habituation includes: 1/ handling for one week to accustom the animals to experimenter touch; 2/ adding the food reward (chocolate cereal Nesquik) in the home cage to habituate the animals to its taste and eliminate hyponeophagia; 3/ scattering the food reward throughout the maze in three consecutive days, devoting 10 minutes to each of the rats to eat the baits and thus to explore the labyrinth.

After the habituation, each rat was subjected to a series of 10 trial sessions daily for three consequent days. Each trial consisted of 2 runnings with 30 s delay between them. At the beginning of each trial, the rat was put in the start arm, and allowed to enter in one of the goal arms (forced) where it received a chocolate pellet (another goal arm is closed by a door). Before the second running, two doors were opened and the rat had a choice to enter in arm that was already visited (incorrect choice) or in another (alternative) arm with a chocolate pellet in the well (correct choice). The identity of the forced goal arm for each trial was determined by random sequences, a different one for each session. Equal numbers of left and right runs were given. Each trial should take no longer than 2 min. To ensure that no odor cues were available, apparatus arms were wiped with 0.1% acetic acid to remove any olfactory clues between trials. Choice accuracy was calculated as the percentage of correct choices.

Corticosterone assay in blood plasma.

After the end of behavioral study, the rats were sacrificed by a rapid decapitation and the trunk blood samples were collected in vacutainers with EDTA as an anticoagulant and stored overnight at 4°C. The probes were centrifuged to remove blood cells and obtain plasma and the concentration of corticosterone was quantified using enzyme-linked immunosorbent assay according to manufacturer protocol (IBL International) using an ELISA reader (ELx800 Absorbance Reader, BioTek Instruments Co.). The samples were collected from 11:00 to 12:00 a.m.

1.4. Statistics.

All data were analyzed by two-way ANOVA (factor DM: Controls and DM; factor Novokinin: saline and Novokinin) or one way repeated measure ANOVA (factor time) and Bonferroni post hoc test. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Study of the influence of novokinin on the weight, urine excretion, water and food consumption in healthy rats and rats with experimental model of T1DM.

Acute intraperitoneal injection of streptozotocin (STZ) at a dose of 65 mg / kg body weight provoked the developement of T1DM in 100% of rats (treated with saline or novokinin) , which was confirmed by the significant increase of plasma glucose concentration, 48 hours after the administration of toxin: T1DM = 28.6 ± 2.99 mmol/l * and T1DM+NVK = 29.72 ± 2.46

mmol/l * vs the healthy Controls = 5.5 ± 0.3 mmol/l. It was registered a drastic rise in mortality in rats with the experimental model of T1DM, that were pretreated i.c.v. with NVK before the induction of T1DM (37.5%) as compared to 0% mortality in saline-treated rats ($p < 0.001$) with T1DM during the period of observation (4 weeks).

The experimental model of T1DM provoked a dramatic and a fast decrease in body weight gain [$F(1, 154) = 394.010$, $p < 0.001$] (Fig. 2A). NVK induced a decrease in weight gain in healthy rats, during the first four days after the beginning of the infusion [$F(1, 168) = 6.986$, $p = 0.009$], however, it was able to diminish DM-induced loss in weight during first 5 days after the injection of the toxin [$F(91, 168) = 31.849$, $p < 0.001$] (Fig. 2A).

STZ-induced DM produced a marked increase in volume of water intake for 24 hours [$F(1, 164) = 464.958$, $p < 0.001$] (Fig. 2B). NVK induced significant increase in water intake from 2-nd to 6-th day after the beginning of the ICV infusion in healthy animals [$F(1, 166) = 100.018$, $p < 0.001$], and potentiated the DM-induced polydipsia [$F(1, 158) = 55.247$, $p < 0.001$] three days after the injection of STZ (Fig. 2B).

NVK caused an increase in the excretion of urine in control animals [$F(1, 166) = 5.830$, $p < 0.001$], as this effect showed time-dependent pattern and disappeared completely after the ninth day of the treatment [$F(1, 166) = 4.362$, $p < 0.001$] (Fig. 2C). The induction of the DM in saline infused rats was accompanied by polyuria, but the group pretreated with NVK showed a statistically significant increase of urine excretion [$F(1, 181) = 44.726$, $p < 0.001$] (Fig. 2C).

The peptide NVK cause a significant increase in food intake in healthy control [$F(1, 169) = 49.539$, $p < 0.001$]. This effect was temporary and disappeared completely seven days after the start of treatment [$F(1, 169) = 3.910$, $p < 0.001$] (Fig. 2D). The induction of DM was accompanied by polyphagia, but the group pretreated with novokinin showed a statistically significant suppression in food intake [$F(1, 181) = 20.708$, $p < 0.001$] compared to the diabetic animals infused with saline. The effect of the peptide was significant only in the first four days after the induction of DM [$F(1, 181) = 26.345$, $p < 0.001$] (Fig. 2D).

3.2. Study of the influence of NVK on the exploratory activity (ambulation and rearing recorded in “open field”), anxiety-like behavior (“elevated plus maze”), working memory (T-maze) and nociception (paw pressure analgesimeter) in healthy rats and rats with an experimental model of DM1.

Chronic treatment with NVK in healthy rats did not change the horizontal and vertical motor activity. The diabetic rats are characterized by a significantly lower ambulation [$F(1,30) = 20.755, p < 0.001$], and rearing [$F(1,30) = 6.397, p = 0.017$] regardless of whether they are treated with saline or NVK in “open field” test (Fig.3A and B).

It should be noted that against the background of the reduced total path traveled, the percentage of the distance in the central area compared to the total path was increased [$F(1,34) = 7.759, p = 0.009$] (Fig. 3 C).

NVK was a factor that induced an anxiogenic -like behavior in rats with DM expressed by the decreased distance traveled in open arms [$F(1, 38) = 6.720, p=0.014$; interaction between NVK and DM: $F(1,38) = 4.332, p=0.048$] and especially by the percent of time spent in open arms [$F(1,38) = 4.253, p= 0.047$; interaction - $F(1,38) = 6.289, p=0.017$]. NVK infused in healthy rats showed a tendency to induce an anxiolytic effect, increasing the time spent in open arms ($p=0.02$ compared to the controls) (Fig.4 A, B).

DM significantly decreased the number of correct alternations in T-maze test [$F(1, 38) = 13.723, p=0.003$ compared to healthy controls], while pretreatment with NVK restored this type of behavior to the control level [$F=6.323, p=0.029$ compared to the DM group] (Fig. 5A).

DM rats showed a hypersensitivity to mechanical pressure [factor DM: $F(1, 38) = 24.280, p<0.001$]. Pretreatment with NVK additionally decreased pain threshold in DM rats [$p=0.033$ vs DM group] potentiating DM-induced hyperalgesia [DM/ NVK interaction $F(1, 38) = 4.249, p=0.049$]. ICV infusion of NVK in healthy rats did not change their nociception (Fig. 5B)

3.3. Study of the influence of novokinin on the level of the corticosterone tested by ELISA in rats with experimental model of DM1.

DM induced a significant increase in plasma level of corticosterone [$H = 7.437, p= 0.004$] and NVK pretreatment did not change the hormone level in diabetic rats (Fig.6).

4. Discussion

In the present study, we found that chronic activation of AT₂ receptors in the brain by a peptide NVK: 1) increased diuresis and water intake in healthy rats without affecting greatly their behavior; 2) amplified DM-induced polyuria, polydipsia and decrease in body weight gain, and increased the mortality rate of rats with DM; 3) accelerated manifestation of behavioral hypo-activity, anxiety and pain hypersensitivity in the early stage of DM development; 4) ameliorated

DM-induced decline in working memory. These results suggest that the brain AT₂ receptors participate in the mechanism of the diabetes mellitus complications occurrence and aggravate some of the DM-induced metabolic and behavioral changes.

Our previous data showed that intracerebroventricularly infused AT₂ receptor agonist CGP 42112A increases water intake six days after the beginning of the treatment and this effect was prolonged and accompanied by an increase in urine output (Pechlivanova and Stoynev 2013). Octapeptide angiotensin II showed a well pronounced dipsogenic effect after ICV injection and this effect depends on activation of both AT₁ and AT₂ receptors in the brain (Li et al. 2003). Earlier data showed that CGP 42112A was able to abolish the antidiuretic effect of centrally infused arginine vasopressin, the result that may explain at list in part the diuretic and dipsogenic effect of the AT₂ receptor agonist (Saad et al. 2005).

Mice brain expressed AT₂ receptors in several brain regions associated with cardiovascular and autonomic regulation, including the rostral ventrolateral medulla (RVLM), subfornical organ (SFO) and also the paraventricular nucleus (PVN), where vasopressin is synthesized (Gao et al. 2012). Moreover, central injection of the octapeptide Ang II increased the level of arginine vasopressin and this effect seems to be regulated mainly by AT₁ receptor, however it is suppressed by the AT₂ receptor (Li et al. 2003). Thus, one of the presumed mechanisms by which NVK increases diuresis and water intake can be related to its inhibitory influence on the vasopressin action. Additionally, vasopressin is involved in the marked increase in urinary albumin excretion observed in diabetes mellitus through increased glomerular leakage. This process requires functional vasopressin V₂ receptors, and is, at least in part, mediated by the renin–angiotensin system (Bardoux et al. 2003).

Our previous data showed that activation of AT₂ receptors in the brain is related not only to an increase of natriuresis but also to a rise in potassium excretion, which suggest their participation in a more complex mechanism regulating water-salt balance, perhaps involving aldosterone (Pechlivanova and Stoynev 2013). The expression of AT₂ receptors in brain structures crucial for the endocrine regulation suggests its role in the control of food intake and metabolic rate. The finding that AT₂R-deficient mice are characterized by adipose tissue with small adipocytes and increased cell number and increase in several genes involved in lipid metabolism in muscles confirms the role of this type of Ang receptor in the metabolism. (Yvan-Charvet et al. 2005). Further, it was found that activation of AT₂ receptors reduced high fat diet-

induced adiposity and improved the lipid metabolism in the liver that resulted in reduced body weight (Nag et al. 2015). It is known that elevated local formation of endogenous angiotensin II induces processes associated with pathogenesis of chronic liver disease through possessing pro-oxidant, fibrogenic, and pro-inflammatory impact in the liver but activation of the alternative axis (ACE2/Ang(1-7)/mas) of the RAS serves as an anti-inflammatory, antioxidant and anti-fibrotic component of the RAS (Ahmadian et al. 2016), but further studies need to establish the role of AT2 receptors in this mechanism. In line with our preceding data obtained after chronic ICV treatment CGP 42112A, NVK also induced an increase in food intake but decreased weight gain in healthy Wistar rats (Pechlivanova and Stoynev 2013). These findings might be important concerning increased risk for metabolic and cardiovascular disorders, especially in people with obesity (Tarantino et al. 2009). Chronic treatment with NVK, however, suppressed the food intake in diabetic rats by an unknown mechanism. The feeding suppression and a additional increase of diuresis during the initial period after the induction of the metabolic disease may be one of the main reasons for the high mortality rate in diabetic rats treated with NVK. These data, however, need further detailed research of the specific mechanisms.

Parallely with its effects on some main metabolic processes our data showed that NVK has a significant impact on the behavioral parameters related to the control of motor activity, exploratory activity, nociception, anxiety and memory. The percentage of time spent in the open arms as an indicator of low level of anxiety, was not changed in DM rats two weeks after the injection of STZ compared to the healthy controls. These data fully confirm previous studies finding that anxiety is reinforced in STZ-injected rats barely four weeks after injection of the toxin (Alba-Delgado et al. 2016). There are some discrepancy between our results and those of Aksu et al. (2012), where they reported for developed anxiety in rats two weeks after the induction of DM. However, they carried out the experiments on female rats that are more vulnerable to DM-induced changes in anxiety and depression (Aksu et al. 2012; Petrov et al. 2013). Current data indicate that NVK causes a mild anxiolytic effect in healthy rats, but it provokes anxiety-like behavior in animals with DM. This underlines the pathophysiological changes caused by the metabolic disease, leading to functional alterations associated with the anxiety-like behavior that were able to reverse the inherent effect of NVK.

Treatment with NVK before and after STZ causes expressed anxiety; thereby the peptide accelerates the onset of this behavioral complication of the metabolic disease. One of the main

reasons for the occurrence of the anxiety-like behavior is the imbalance between the activities of brain monoaminergic mediator systems. It was found that in rats with DM-induced anxiety, the activity of neurons in the LC is significantly reduced and that this process is controlled by the α_2 -adrenoreceptor (Alba-Delgado et al. 2016). AT₂ receptors have a high density in this cerebral structure suggesting their role in the regulation of central norepinephrine activity (Tsutsumi and Saavedra 1991). Recently published data confirmed this suggestion, as the infusion of a peptide AT₂ receptor agonist in the brain was able to decrease the NE release in the hypothalamus caused by electrical stimulation in the LC (Gong et al. 2015). According to others, our result confirmed that uncontrolled DM induced a significant elevation of corticosterone level in blood plasma as a result of an activation of the hypothalamic-pituitary-adrenal (HPA) axis (Schwartz et al. 1997). This activation of the HPA axis by diabetes is associated with a proportionate decrease in hypothalamic paraventricular nucleus (PVN) of the corticotrophin-releasing hormone (CRH) gene expression by negative glucocorticoid feedback. Besides these hormonal changes, STZ-induced DM was accompanied by decreased serotonergic turnover, impaired normal response to acute stress and anxiety-like behavior (Thorre et al. 1997).

One of the most common complications associated with DM is diabetic neuropathic pain, which is manifested by increased sensitivity to pain stimuli. Our present results are in concert with literature data reporting an increased susceptibility to paw pressure in rats after two weeks of DM induction (Ahlgren and Levine 1993). We have reported that single intracerebral injection of AT₂ receptor antagonist produced a short lasting pro-nociceptive effect in a model of visceral pain in mice that suggested an antinociceptive effect of this receptor subtype in healthy mice (Georgieva and Georgiev 1999). Further study on the effects of AT₂ receptor agonist CGP 42112A on the nociception in rats showed that acute intracerebral injection induced a short lasting antinociception, however, after chronic ICV infusion the agonist produced a pronociceptive effect in particular time points of the diurnal cycle (Pechlivanova et al. 2013). In the present study NVK alone did not change the pain threshold at the particular time point; however, its infusion in diabetic rats increased the diabetes-induced hyperalgesia. Recently published study revealed the participation of AT₂ receptors in neuropathic pain due to mechanism of peripheral pain sensitization of DRG in humans (Anand et al. 2015).

What is the mechanism of these opposite effects of AT₂ receptor activation in view of acute and chronic scheme of treatment? We have to keep in mind that the endogenous

angiotensin II is able to bind with both AT1 and AT2 receptors and to accomplish diametrically opposite effects. Moreover, chronic treatment with some receptor ligand can up- or down-regulate variety of receptors those capable of binding to this ligand. For example, sustained peripheral administration of an AT2 antagonist decreases binding to brain AT2 receptors, however, it up-regulates AT1 receptors expression in specific brain areas (Macova et al. 2009). Additionally, recently reported data corroborate that SZT-induced diabetes in mice significantly increased the expression of angiotensin II and ACE at the level of lumbar dorsal spinal cord and induced allodynia that was abolished by an AT1 receptor antagonist (Ogata et al. 2016). Thus, chronic treatment with NVK might induce plastic and functional changes leading to aggravation of diabetes-induced hyperalgesia.

Streptozotocin-induced diabetes resulted also in deleterious effects on memory formation, particularly impairment of hippocampus-dependent spatial memory. Cognitive dysfunction in diabetic rats seems to be a result of increased oxidative stress, increased acetylcholinesterase activity (Schmatz et al. 2009) and iNOS induced NO production in brain (Zhou et al. 2017). Our present data confirmed the deleterious effect of diabetes on the spatial memory expressed by decreased alternation in the T-maze test. One of the positive effects of the NVK treatment established in this study was attenuation of diabetes-induced impairment of the spatial memory. There are several reports on the memory protective effects of other AT2 receptor agonists (CGP 42112A and Compound 21) in experimental focal reperfusion model of stroke injury, diabetes type 2 and Alzheimer disease (McCarthy et al. 2009; Jing et al. 2012; Iwanami et al. 2014). The authors suggested that the protective effect of AT2 receptor activation is related to bradykinin/B2 receptor/NO pathway dependent vasorelaxation and improved brain blood flow as well reduced oxidative stress and potentially associated neuronal apoptosis.

AT2 receptor seems to participate in a wide variety of brain-controlled functions including metabolic processes, pain susceptibility, motor activity and spatial memory. Chronic intracerebral treatment with a selective receptor agonist is prone to aggravate some of diabetes induced complications but improved spatial memory. Despite the limited capabilities of peptides such as Novokinin to penetrate the blood-brain barrier and to reach to brain structures controlling metabolism and behavior, there is still a potential risk of using the peptide as an antihypertensive agent in diabetic patients to cause or aggravate complications of the metabolic disease.

These initial data suggest that angiotensin AT2 receptors participate in the pathogenesis of DM-induced complications in the function of the nervous system. The AT2 receptor agonist Novokinin infusion in the cerebral ventricles exacerbated diabetes-induced hyperalgesia and provoked anxiety-like behavior but improved spatial memory in diabetic rats.

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Figures captures

Fig. 1 Experimental design of the study. Novokinin was infused at a dose of 0.6 $\mu\text{g}/\text{rat}/\text{day}$ for 28 days through osmotic minipumps; STZ – streptozotocin was injected once at a dose of 65 mg/kg, intraperitoneally; OF – Open field test; EPM – Elevated plus maze test; PP – Paw pressure test; T-maze test.

Fig. 2 Effects of the AT₂ receptor agonist Novokinin (0.6 $\mu\text{g}/\text{rat}/\text{day}$, 28 days) and induction of type 1 diabetes mellitus (14 days after 65 mg/kg STZ, IP) on the rate of daily body weight gain (A), 24 hour water intake (B), 24 hour urine excretion (C) and 24 hour food intake (D) to 100 grams body weight. Data presented as mean $\pm$ SEM, n= 10. * P <0.05 vs controls infused with saline.

Fig. 3 Effects of the AT₂ receptor agonist Novokinin (0.6 $\mu\text{g}/\text{rat}/\text{day}$, 28 days) and induction of type 1 diabetes mellitus (14 days after injection with streptozotocin) on the ambulation (total distance traveled) (A), the rears (number of rises on the rear paw) (B), open vs total distance traveled ratio (in percentages) (C) in test “open field”. Data presented as mean $\pm$ SEM, n= 10. * P <0.05 vs controls infused with saline; # p<0.05 vs healthy rats infused with Novokinin.

Fig. 4 Effects of the AT₂ receptor agonist Novokinin (0.6 $\mu\text{g}/\text{rat}/\text{day}$, 28 days) and induction of type 1 diabetes mellitus (14 days after injection with streptozotocin) on the state of anxiety: distance traveled in the open arms compared to the total distance presented in percents (A) and percents of time spent in the open arms (B) studied with test "Elevated PlusMaze". Data presented as mean $\pm$ SEM, n= 10. ^op <0.05 vs DM rats infused with saline.

Fig. 5 Effects of the AT₂ receptor agonist Novokinin (0.6 $\mu\text{g}/\text{rat}/\text{day}$, 28 days) and induction of type 1 diabetes mellitus (14 days after injection with streptozotocin) on the working memory represented by Choice accuracy in percents, using T-maze rewarded alternation test (A); and on nociception represented by the withdrawal threshold in grams studied with test "Paw

pressure" (B). Data presented as mean $\pm$ SEM, n= 10. * $p<0.05$ vs controls infused with saline; # $p<0.05$ vs DM rats infused with saline.

Fig.6 Effects of the AT2 receptor agonist Novokinin (0.6 μ g/rat/day, 28 days) and induction of type 1 diabetes mellitus (14 days after injection with streptozotocin) on the corticosterone levels in blood plasma tested by ELISA. Data presented as mean $\pm$ SEM, n= 10. * $p<0.05$ vs controls infused with saline.

Draft

Open Field

Total distance traveled

Open field

Vertical activity (rearing)

C

Open field

central / total distance traveled

A

Elevated Plus Maze

open arms/ total distance

B

Elevated Plus Maze

time spent in open arms/ total time