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THE AMELIORATIVE EFFECTS OF AT2 RECEPTOR ACTIVATION WITH THE HEXAPEPTIDE NOVOKININ ON STREPTOZOTOCIN-INDUCED MODEL OF ALZHEIMER'S DISEASE IN SHR

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Abstract

Alzheimer's disease (AD), a form of dementia, presents a global concern. This study investigates the connection between hypertension and AD and the effects of the peptide novokinin (NVK), an AT2 receptor agonist in spontaneously hypertensive rats (SHRs). The brain's renin-angiotensin system (RAS) influences cognitive and emotional processes, suggesting potential intersections with AD. Streptozotocin (STZ), administered intracerebroventricularly (ICV), is used as a model of late-onset Alzheimer's disease. This model reveals a memory impairment associated with an insulin-resistant brain state, marked by neuroinflammatory processes contributing to cognitive deficits, oxidative stress, and reduced cerebral energy metabolism. NVK is an angiotensin AT2 receptor agonist, studied for its potential neuroprotective role. Female SHRs were used to investigate the effects of STZ-ICV (twice) and chronic NVK-ICV. NVK was administered for 14 days ICV using osmotic minipumps (ALZET®). The control group exhibited strain-typical increased exploratory activity, reduced anxiety in new environments, and impaired spatial memory, as expected for SHRs. The STZ-ICV negative control group showed signs of impaired memory and object recognition. In the 'Open Field' test, STZ-treated groups with and without NVK-ICV displayed heightened exploratory behaviour. Data from

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the 'Elevated plus maze' test showed decreased anxiety in the STZ-ICV group while combining STZ and NVK normalized the anxiety behaviour. In the 'T-maze' test, STZ-ICV-treated rats made fewer correct decisions. The STZ+NVK group exhibited enhanced spatial memory and improved anxiety-like behaviour in novel environments, potentially attributable to the proposed neuroprotective effects of NVK. These findings emphasize the association of RAS in the progression of AD and the potential protective role of the brain's AT2 receptors in this type of dementia.

Key words: Alzheimer's disease, novokinin, streptozotocin, spontaneously hypertensive rats, anxiety, memory

Introduction. Alzheimer's disease is the predominant cause of dementia, currently posing a significant worldwide concern. Recent findings emphasize hypertension's role in AD pathogenesis. Amyloid β-peptide (Aβ) accumulation initiates entorhinal cortex-originated neurotoxic cascades in AD, involving multiple neuron regions. The second AD histopathological hallmark is hyperphosphorylated microtubule-associated protein Tau-induced neurofibrillary tangles, impacting axon terminal signalling and dendritic nutrition [1]. Streptozotocin (STZ), a naturally occurring compound derived from *Streptomyces achromogenes*, has been utilized via intracerebroventricular injection, as a model of late-onset AD accompanied by memory impairment, neuroinflammatory processes, oxidative stress, diminished cerebral energy metabolism, Aβ deposition, and hyperphosphorylation of tau protein in the hippocampus [2].

The renin-angiotensin system (RAS) participates in homeostasis by controlling blood pressure, plasma sodium level, inflammation, cell proliferation, fibrosis, and oxidative stress [3]. The RAS consists of a cascade starting with renin released into the circulation from the kidney, where it cleaves angiotensinogen to form angiotensin I (Ang I). Ang I can be converted by angiotensin-converting enzyme 1 (ACE1) to angiotensin II (Ang II) or by angiotensin-converting enzyme 2 (ACE2) to angiotensin 1-9 (Ang1-9) [4]. RAS effects on the target organs are realized through angiotensin receptors AT1, AT2, AT4, and Mas receptor (MasR). Ang II is an AT1 and AT2 receptor agonist with similar affinity to both receptors. AT1 receptor activation is related to hypertension, vascular remodelling, endothelial dysfunction, and organ damage [4]. AT2 receptors counteract the action of AT1 receptors. Recently it has been shown that the MasR also counter-regulates AT1 receptor effects, and together with AT2, they are the so-called protective "non-classic" balancing arm of RAS [5–7]. Moreover, female mice have shown a decreased AT1/AT2 ratio which was suggested to contribute to the protective effects of estrogen [8]. The brain has its own RAS that modulates sensory information, emotional and behavioural responses, nociception, stress responses, anxiety, learning, and memory. In adults, AT2 receptor expression is limited to specific brain areas, including the medulla oblongata, septum, amygdala (linked to anxiety-like behaviour), thalamus, superior colliculus, subthalamic nucleus, and

cerebellum. AT2 receptor expression was found also in the substantia nigra pars compacta, and the hippocampus. In AD, the temporal cortex in the adult brain exhibits increased expression of the AT2 receptor, while the hippocampus shows decreased expression. This pattern of expression aligns to comprehend the roles of AT2R [9].

Novokinin (NVK) is a synthetic hexapeptide derived from ovokinin, which is investigated for its potential therapeutic effects, particularly as an AT2 receptor agonist. In recent years novokinin is investigated for its potential neuroprotective, cognitive, anxiety, and memory effects. As research progresses, novokinin's role in modulating the RAS within the brain continues to show promise, offering a potential novel therapeutic approach for treating conditions like AD that involve both vascular and neurodegenerative components [10].

This study aimed to reveal the relationship between brain AT2 receptor activation by the peptide NVK and impaired RAS in female spontaneously hypertensive rats (SHR) with an experimental model of Alzheimer's disease induced by intracerebroventricular streptozotocin (STZ-ICV).

Materials and methods. ***The surgical procedure.*** A total of 24 female SHRs, 8 weeks of age at the beginning of the experiments were included. ICV injection cannulas were stereotaxically implanted bilaterally into the lateral brain ventricles, guided by coordinates AP = −1 mm, L = 1.6 mm, and DV = −4 mm, according to the rat brain in stereotaxic coordinates. The procedure was conducted under anesthesia using Ketalar (100 mg/kg, i.m.) and Xylazine (5 mg/kg, i.p.). The cannulas were secured to the skull using screws and dental cement, a pocket was created between the scapulae for an osmotic mini-pump, followed by wound closure with sutures. SHRs were administered ICV injections of streptozotocin, twice, at a dose of 3 mg/kg body weight. After STZ injection, NVK was introduced directly to the lateral ventricle at a dose of 0.3 µg/rat/day over two weeks using osmotic minipumps (ALZET®, Cupertino, CA, USA, model 2002). These pumps operate at a rate of 0.50 µL per hour and were connected with brain kit 2 (also from ALZET®, Cupertino, CA, USA). The control group underwent the same insertion process (sham-operated) but was only given saline. The rats were housed under standard laboratory settings with access to rat food pellets and unlimited tap water. Behavioural assessments were subsequently conducted 3 months post-STZ injection. All animal experiments were carried out following the Declaration of Helsinki Guiding Principles on Care and Use of Animals, EC Directive 2010/63/EU, and were approved by the Ethics Committee of the Bulgarian Food Safety Agency (No. 389/2029).

"Open Field" test. Each animal was placed at the centre of an opaque enclosure, measuring 100 × 100 × 60 cm. Behavioural tracking was facilitated by the SMART video tracking system (Panlab, Harvard Apparatus) over a duration of 5 min. For each subject, trajectory lengths in the peripheral and central zone (60 × 60 cm) were documented at 1-minute intervals throughout the assessment [11].

***"Elevated* Plus Maze" test (EPM).** The apparatus incorporated two open arms (50×10 cm), two enclosed arms ($50 \times 10 \times 40$ cm), and a central platform (10×10 cm), all elevated 50 cm from the floor. Rats were individually positioned on the central platform facing an open arm and monitored for 5 min. Parameters such as the total trajectory travelled, the ratio of open arms to the total trajectory, and the proportion of time spent in open arms relative to the total observation time were captured and computed using the SMART video tracking system (Panlab, Harvard Apparatus).

"T-maze rewarded alternating" test. The apparatus consisted of a start arm ($42 \times 11.4 \times 11.4$ cm) connected to two goal arms with doors. Before training, rats underwent restricted feeding and habituation. The procedure involved ten daily trials over three days. Each trial had two runs with a 30-second delay. In the first run, the rat accessed the arm with a food pellet, while the other arm was blocked. In the second run, both arms were open, and the rat could either revisit the previous arm (incorrect) or explore the other arm with a new reward (correct). Accuracy was measured by the proportion of correct choices.

"Novel Object recognition" test. The experimental apparatus was an opaque box measuring $50 \times 50 \times 60$ cm. The procedure had three phases: a 15-minute habituation in the empty box, a 5-minute training phase with two identical objects, and a 5-minute testing phase 15 min later, where rats explored a familiar and a novel object. Preference for the novel object was measured using a Recognition Index.

Statistics. Data were subjected to statistical analysis using ANOVA with STZ-ICV and NVK treatment as the factors, followed by post-hoc comparisons using the Tukey test. $P < 0.05$ was deemed statistically significant.

Results. ***"T-maze" test.*** The SHR-STZ group, exhibited Alzheimer's-like symptoms showing impaired learning, decision-making, and memory, making significantly fewer correct alternations on the first and second days.

The SHR-STZ+NVK group displayed significantly more correct alternations ($p < 0.05$) on the first and third days showcasing an improvement in working and spatial memory and habituation in a novel environment (Fig. 1).

"Open Field" test. SHR-STZ and SHR-STZ+NVK groups displayed a significant ($p < 0.05$) increase in the exploratory behaviour, against the control group (Fig. 2A). No significant differences were found between groups in terms of activity in the central zone (Fig. 2B).

"Elevated Plus Maze" test. The control and SHR-STZ groups are presented with reduced anxiety-like behaviour. On the contrary, the SHR-STZ+NVK group spent significantly less time in the open arms ($p < 0.05$), close to normal anxiety-like behaviour in a new environment (Fig. 3).

"Novel Object Recognition" test. No statistical significance between the tested groups was noted (data not shown).

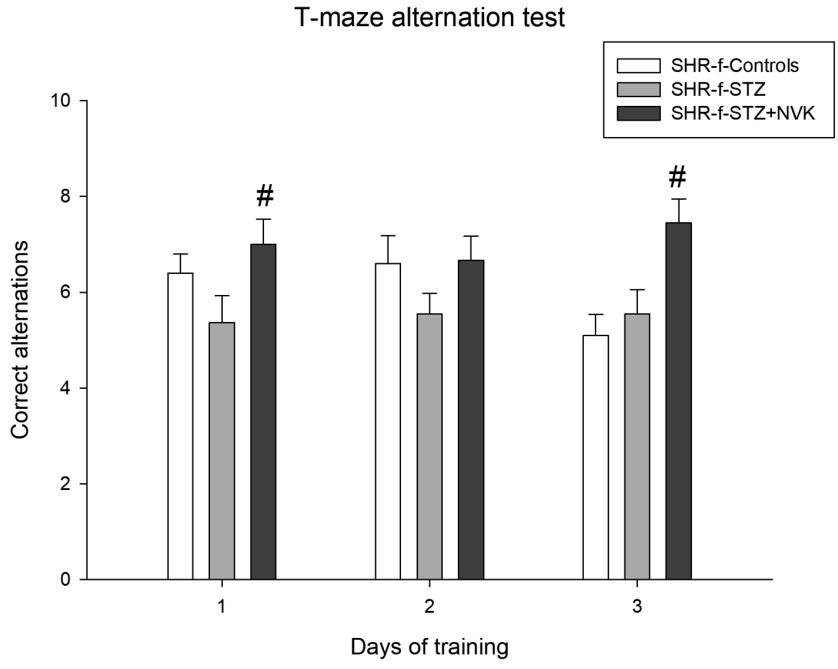

Fig. 1. Effects of NVK on the spatial memory in the "T-maze alternation" test in SHRs. The STZ+NVK group showed a statistically significant increase in the correct alternations on days one and three of training, compared to the STZ group ($F = 13.778$, $\#\ p < 0.001$), $n = 8$

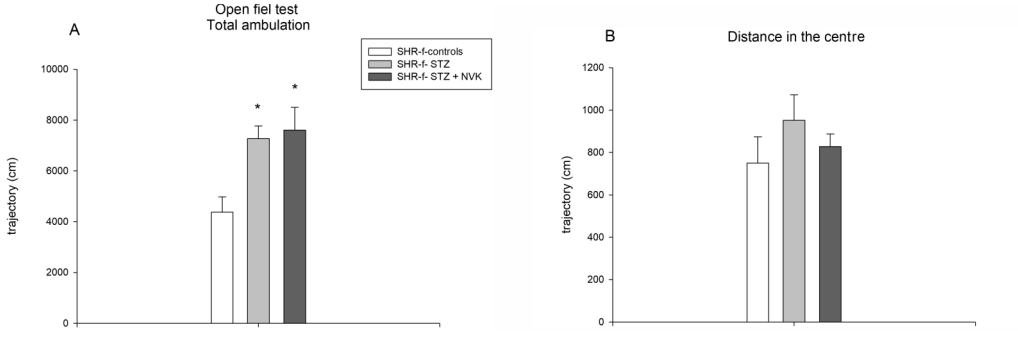

Fig. 2. A. STZ-ICV-induced hyperactivity (total ambulation) in test "Open field" in SHR ($F = 5.903$, $^{*}p = 0.01$ compared to the Controls) showing an increased exploratory behaviour. There is no significant difference between STZ-ICV and STZ-ICV+NVK. B. There was no significant difference between the tested groups in the motor activity in the central aversive area of the "Open field" ($F = 0.937$, $p = 0.409$), $n = 8$

Discussion. The main findings in this study demonstrate that infusion of the AT2 receptor agonist NVK into the brain of female SHR normalizes anxiety-like behaviour in a novel environment (in the elevated plus maze) and improves hippocampus-dependent spatial working memory (in T-maze). Despite these ame-

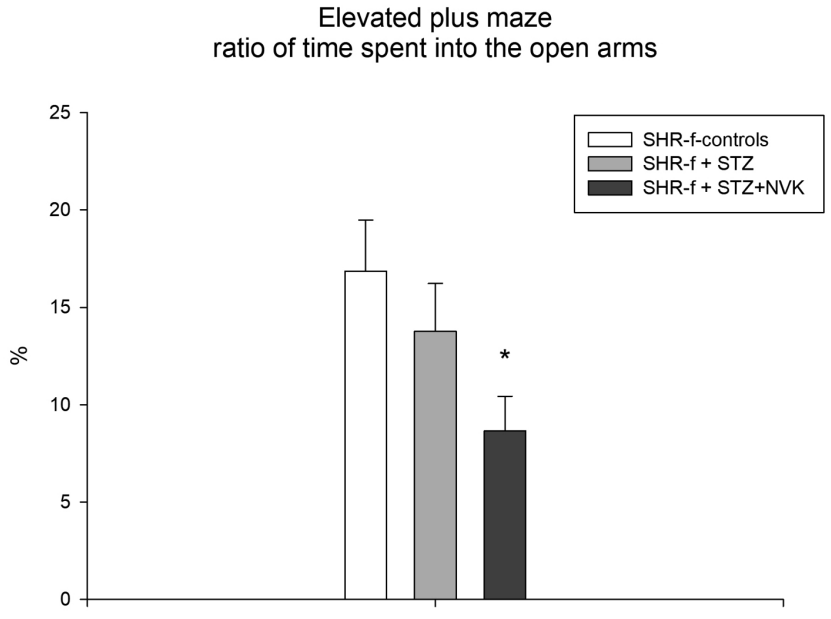

Fig. 3. Effects of STZ and NVK treatment on the anxiety-like behaviour in the "Elevated plus maze" test in SHR. The STZ+NVK group showed a statistically significant decrease in the ratio of time spent in the open compared to the Controls ($F = 6.369$, $^*p = 0.020$) but no significant difference to the STZ-NVK group ($F = 2.747$, $p = 0.081$), $n = 8$

liorative effects, peptide treatment did not affect STZ-induced hyperactivity (in the open field) and impaired object-recognition working memory.

SHR is characterized by the development of essential hypertension as well as some specific behavioural abnormalities such as hyperactivity, impaired anxiety, and memory, making this rat strain a suitable model for studying attention deficit hyperactivity disorder [12,13]. Notably, hypertension's involvement is not limited to vascular dementia but extends to AD pathogenesis, which was traditionally viewed as a primarily neurodegenerative condition [13]. On the other hand, evidence of increased brain activity in the SHR was detected in areas such as the anterior hypothalamus, supraoptic nucleus (SON), locus coeruleus, and pedunculopontine tegmental nucleus which are linked with system arousal, cardiovascular regulation, and motor function [14,15]. Based on the literature and our research, it has been determined that SHRs exhibit a pronounced decline in their learning and memory functions compared to normotensive rats [10,16]. The above brain regions expressed a high density of AT2 receptors as previously shown, consistent with the involvement of AT2 receptors and RAS imbalance in the development of behavioural abnormalities in SHR. Moreover, the renal hypertensive rats demonstrated upregulated expression of AT1 and AT2 receptors in the hippocampus [17]. Our recent data showed that NVK treatment via chronic brain infusion

in SHRs provokes a diverse range of positive effects. It normalized SHR-specific behavioural parameters associated with hyperactivity and anxiolysis, heightened pain threshold, and improved adaptation to new environments [10]. The present data highlight the positive tendency of NVK to normalize anxiety-like behaviour in SHRs with comorbid AD (Fig. 3). Notably, the observed enhancement in spatial memory in the T-maze test (Fig. 1) during prolonged NVK treatment aligns with the intricate neural patterns associated with AT2 receptors and our preview data [10]. Chronic ICV infusion of NVK showed some adverse effects in an animal model of DM in terms of aggravation of diabetes-induced disturbances in metabolic control, however, among the positive effects of the peptide was the significant improvement of spatial memory in diabetic normotensive rats [11].

The present study found no significant effect of NVK treatment on object recognition memory impairment (novel object recognition test) and spontaneous hyperactivity (open field test) in SHR. It is important to note that recognition memory involves recollection and familiarity, which are the main components of distinguishing novel from familiar objects [18]. Although still a matter of debate, neuroscientists have accepted that the hippocampus and the perirhinal cortex are the two main brain structures responsible for these components. As a complex and dynamic process, recognition memory involves multiple neural structures such as thalamus, hypothalamus, prefrontal cortex, parietal cortex, retrosplenial cortex, and parahippocampal cortex, which support neural interconnections forming object discrimination [18].

Despite the poor understanding of AT2R, its potentially beneficial effects in neuroprotection extend beyond neurons to impact blood circulation. Recent studies have shown the AT2 receptor's protective role in response to brain ischemia, reducing damage and restoring cognitive function. This multifaceted potential of AT2 receptor activation is further highlighted by its influence on reducing apoptosis signalling and regulating the phosphorylation of the microtubule-associated protein tau, a crucial factor in AD development. Collectively, these findings imply that the AT2 receptor may play a crucial role in sustaining the functions of the human brain, connecting its physiological role with potential implications in neurological conditions like AD [19, 20].

Conclusions. In summary, our study demonstrates that AT2 receptor agonist NVK shows promising ameliorating effects in a rat model of streptozotocin-induced Alzheimer's disease with comorbid hypertension. The findings highlight NVK's potential to improve spatial memory and normalize anxiety-like behaviour, suggesting a novel therapeutic approach for addressing both the neurodegenerative and vascular components of AD. However, further research is needed to confirm these effects and fully understand the underlying mechanisms before considering clinical applications.

Note. Filippos S. Chelmis, Iliana N. Sorotou, and Paraskevas E. Pakataridis contributed equally to the article preparation.

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