

EVIDENCE FOR BIOLOGICAL EFFECTS OF THE HEXAPEPTIDE NOVOKININ

Paraskevas E. Pakataridis^{1*}, Filippos S. Chelmis^{1*}, Borislav V. Assenov^{1,2},
Elena B. Dzhambazova¹, Daniela M. Pechlivanova^{1,2}

¹Faculty of Medicine, Sofia University "St. Kliment Ohridski"
1 Kozyak Str., 1407 Sofia, Bulgaria

²Institute of Neurobiology, Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 23, 1113 Sofia, Bulgaria
E-mail: pechlivanova@yahoo.com

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ABSTRACT

The imbalance of the endogenous renin-angiotensin system is one of the mechanisms involved not only in the hypertension but also in the development of behavioral abnormalities. Activation of angiotensin AT2 and Mas receptors is considered a protective and balancing arm in the renin-angiotensin system, and the synthesis and characterization of selective agonists of these receptors is a promising direction for chemical and pharmacological research.

The aim of the present study was to elucidate the effects of intracerebroventricular infusion of the hexapeptide Novokinin, a selective angiotensin AT2 receptor agonist, on anxiety, exploratory behavior, nociception, working and spatial memory in spontaneously hypertensive female rats.

The following battery of tests was used: Open Field and Elevated Plus Maze to study exploration and anxiety-like behaviors, object recognition, T-maze for working and spatial memory, and paw pressure for nociception. Novokinin was infused intracerebroventricularly using osmotic minipumps and a stereotaxically implanted brain kit in adult female spontaneous hypertensive rats. All tests were performed twice: at the end of the infusion and three months later. The rats are characterized by hyperactivity, reduced levels of anxiety and impaired spatial memory, which are associated with the strain's inherent impulsive behavior. Novokinin-treated rats showed reduced anxiety and hyperactivity as well as increased pain threshold compared to controls. The peptide improves adaptation to the new environment and spatial memory without changes in novel object recognition.

In summary, chronic stimulation of brain AT2 receptors by the peptide novokinin improves cognitive behavior and memory and normalizes anxiety-like behavior in spontaneously hypertensive female rats.

Keywords: peptide, angiotensin type 2 receptors, anxiety, memory, nociception.

INTRODUCTION

The synthesis of more selective peptide analogs with conserved pharmacophores for the target receptor is a promising research tendency. Analysis of various Ang II-mimicking peptides indicates a crucial role of residue 6 in the Ang II molecule in enhancing their selectivity to the AT2 receptor [1]. Novokinin (Arg-Pro-Leu-Lys-Pro-Trp; NVK) is a hexapeptide that was synthesized by Fmoc strategy [2]. It is based on Novokinin (2-7) (Arg-Ala-Asp-His-Pro-Phe), a peptide with significant vasorelaxant activity derived from ovalbumin [2 - 4].

Previous studies have shown that the hypotensive, anorexic, and anxiolytic effects of orally administered NVK involve specific activation of an AT2-dependent mechanism [2 - 5].

The renin-angiotensin-aldosterone system (RAAS) participates in homeostasis by controlling blood pressure, plasma sodium level, inflammation, cell proliferation, fibrosis, and oxidative stress [6, 7]. The "classical" RAAS consists of a cascade initiated by renin released into the circulation from the kidney, where it cleaves secreted by the liver 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) and further by ACE to angiotensin 1-7 (Ang1-7) [8]. RAAS mediates its effects through angiotensin receptors which include the AT1, AT2, AT4 and the 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 remodeling, endothelial dysfunction, and organ damage [6, 7]. AT2 receptors counteract the action of AT1 and has vasodilatory effect, produces apoptosis, anti-proliferation, regeneration and provokes differentiation. In the recent years it has been shown that the MasR also counter regulates AT1 receptor effects, and together with AT2 they are so-called protective “non-classic” balancing arm of RAAS [9 - 11]. The brain has its own RAS that modulates sensory information, emotional and behavioral responses, nociception, stress responses, anxiety, learning and memory [12, 13]. Spontaneously hypertensive rats (SHRs) are strain that develop spontaneously arterial hypertension, hyperactivity, reduced anxiety-like behavior, cognitive impulsiveness, and deficient response to re-engagements compared to normotensive Wistar-Kyoto rats [14 - 20]. Accumulating evidence suggests that the non-classical RAS is elevated in females compared to male SHR with a reduced AT1/AT2 receptor ratio, making females more susceptible to AT2 ligands treatment [21 - 23].

The aim of the present study was to elucidate the effects of intracerebral infusion of NVK on anxiety and exploratory behavior, nociception, working and spatial memory in female SHRs.

EXPERIMENTAL

Animals and drug treatments

Novokinin (L-arginyl-L-prolyl-L-leucyl-L-lysyl-L-prolyl-L-tryptophan; NVK, Sigma Aldrich) was dissolved in sterile saline and infused intracerebroventricularly (ICV), at a dose of 0.3 µg/rat/day for 14 days by osmotic minipumps (Alzet, Cupertino, CA, USA, model 2002) which deliver at 0.50 µL h⁻¹ connected with brain kits 2 (Alzet, Cupertino, CA, USA). The Controls received the same implantation procedures (sham-operated) but received saline. The experiments were performed on 2-month-old female spontaneously hypertensive

rats (SHR) with 220 - 280g weight kept in standard laboratory conditions, regular rodent diet and tap water *ad libitum*. Animals were anesthetized (Ketalar, 100 mg kg⁻¹, i.m., Xylazine 5 mg kg⁻¹) and implanted with pumps connected to a brain kit using a stereotaxic apparatus with coordinates 1.5 mm lateral (right) to the sagittal suture, 1 mm caudal to bregma, and a depth of 3 mm. All experiments were approved by Bulgarian Food Safety Agency No 330/2021, which is in accordance with EC Directive 2010/63/EU for animal experiments.

“Open field” (OF) test

The procedure is described in details elsewhere [5, 24], and consisted of positioning an animal in the center of an opaque box (100 X 100 X 60 cm) provided with SMART video tracking system (Panlab, Harvard Apparatus) for 5 min. For each rat, the length of the trajectories in the peripheral and central (60x60 cm) zones were recorded each 1 min of the test.

“Elevated plus maze” (EPM) test

The apparatus consists of two open arms (50 × 10 cm), two enclosed arms (50 × 10 × 40 cm), and a central platform (10 × 10 cm) 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 and calculated [25].

“Paw pressure” test

The mechanical pressure (in arbitrary units) required eliciting pain responses such as withdrawal or struggle was measured with an analgesimeter (Ugo Basile, Italy). The mechanical nociceptive threshold testing was optimized by single training of the animals one day before the experiments [26].

“T-maze rewarded alternating” test

The apparatus (Columbus, USA), consisted of a start arm (42 x 11.4 x 11.4 cm) connected perpendicularly with two identical goal arms, fitted with doors, through a central area. Prior to training, rats were maintained on a restricted feeding schedule and a habituation procedure. Each rat underwent a series of 10 trial (2 runs with a 30 s delay) sessions daily for three consecutive days. On the first running the rat was allowed to enter in one

of the goal arms (baited) 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). Choice accuracy was calculated as the percentage of correct choices [27].

“Novel object recognition” test

The apparatus consisted of an opaque box (50 cm × 50 cm x 60 cm). The procedure includes three phases: (1) habituation to the empty box (15 min); (2) training - exploration of two identical new objects for 5 min; (3) testing - 15 min after the training the rats explore two objects (one familiar and one novel) for 5 min. Discrimination of the novel object was represented by a Recognition index [28].

Statistics

All data were analyzed by ANOVA (factor NVK treatment) and Tukey post hoc test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In the present study, we investigated the effects of intracerebroventricular infusion of NVK, an AT₂ receptor agonist, on exploratory and anxiety-like behavior, acute nociception and two types of memory: referent memory for object recognition and spatial memory in female SHRs. SHRs are characterized by a hyperactivity and impaired anxiety, which shapes their complex impulsivity and attention-deficit [16 - 19, 29]. The Controls SHR showed habituation to a new environment expressed by gradually decrease of the exploratory behavior in the OF test ($F_{4,39} = 9.297$, $p < 0.001$). NVK improved the habituation (13th day $F_{4,39} = 29.778$; 3rd month $F_{4,39} = 25.283$; $p < 0.001$ vs Controls) (Fig. 1(A)). Given that SHRs have inherent hyperactivity and anxiolysis, NVK normalized their locomotor activity 3 months after the treatment ($F_{1,15} = 7.772$, $p = 0.015$; Fig. 1(B)) and anxiety-like behavior reducing the activity in the center ($H = 7.411$, $p = 0.025$; Fig. 1(C)). NVK significantly reduced the anxiolysis in EPM test 3 months after the treatment ($F_{1,15} = 12.388$, $p = 0.003$; Fig. 2 (A, B)) and increased the pain threshold on both the treated ($F_{1,15} = 103.234$, $p < 0.001$) and

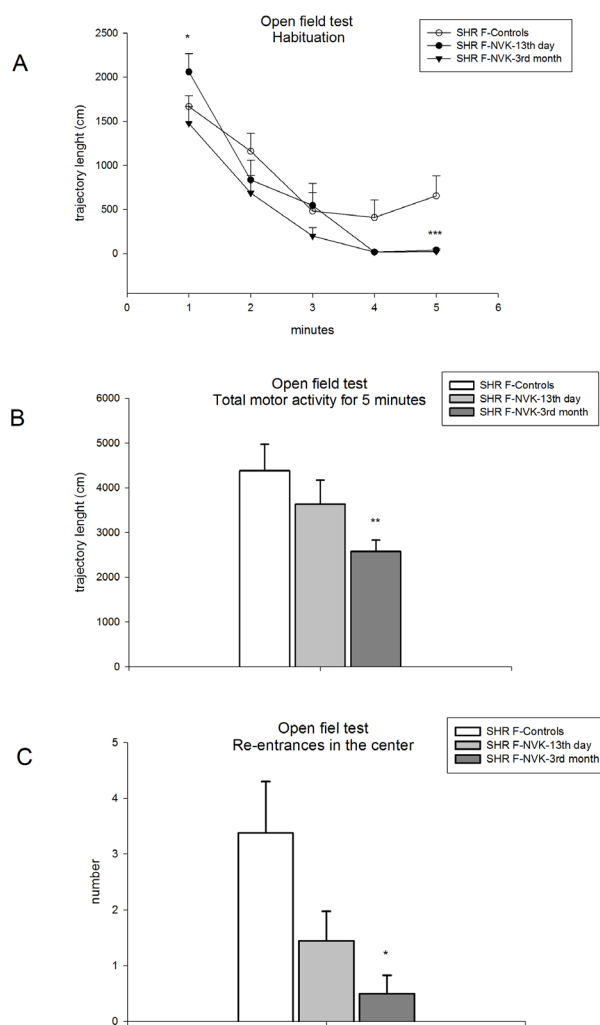


Fig. 1. Effects of the AT₂ receptor agonist NVK on the exploratory behavior in a novel environment. Data from the “Open field” test showed the habituation expressed as a time-dependent decrease in distance traveled in the open field (A), exploratory behavior (total motor activity) (B), and anxiolysis expressed as re-entrances in the center (C) in female SHRs at the end of the peptide ICV infusion (13th day) and in post-treatment period (3rd month). Data are expressed as mean ± SEM (n=8). * $p < 0.05$ vs. saline-treated control group.

post-treated groups ($F_{1,15} = 72.291$, $p < 0.001$) (Fig. 3). The results from OF and EPM tests indicated that NVK treatment reduced hyperactivity and anxiolytic behavior in SHR. AT₂ receptors are distributed in specific brain areas involved in the control of motor activity, the sensory systems, and selected limbic structures related to emotional control as well as in structures of autonomous

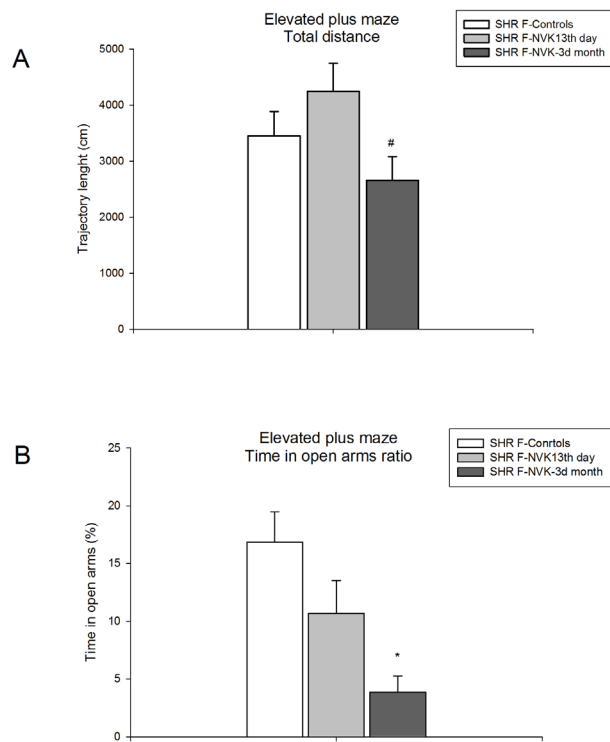


Fig. 2. Influence of the peptide AT2 receptor agonist NVK on anxiety behavior. Data are expressed as distance traveled in the open arms (A), and the ratio of time spent in the open arms (B) in the “Elevated plus maze” test in female SHRs at the end of the peptide ICV infusion (13th day) and in the post-treatment period (3rd month). Data are expressed as mean \pm SEM (n = 8). *p < 0.05 vs. saline-treated control group; # p < 0.05 vs. 13th day of the infusion.

functions regulation [11, 12]. Most of these areas was recently found to be less active in SHRs as compared to normotensive rats [19]. Intracerebral NVK infusion produced a significant and long-term antinociception in female SHRs. Our earlier data showed that blockade of brain AT2 receptors produced a pronociceptive effect in a visceral pain model [30]. Further, we have shown that the partial AT2 agonist was able to induce antinociception after acute treatment, whereas in a chronic regimen it can reverse this effect [31]. We can speculate that the stronger antinociceptive effect of NVK in female SHRs is a result of their increased non-classical RAS and reduced ratio of AT1/AT2 receptors, making females more susceptible to treatment with AT2 ligands [1, 2, 22, 23]. Despite growing evidence for AT2 receptor ligands as potential analgesics, the role of AT2 receptors

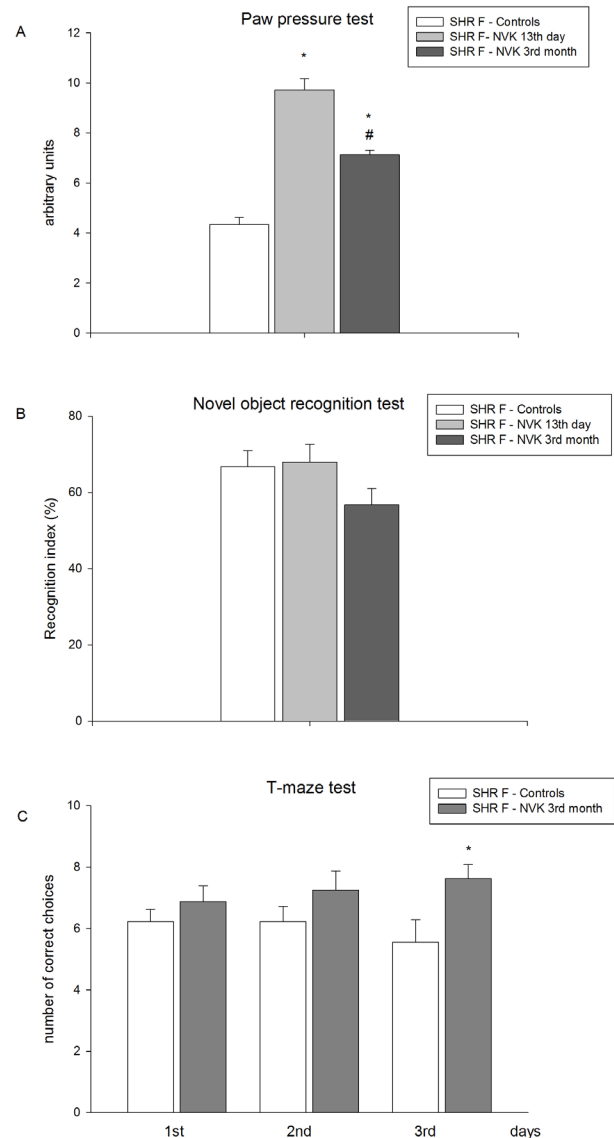


Fig. 3. Effects of the AT2 receptor peptide agonist NVK on the nociception (A), recognition memory (B), and spatial memory (C) in female SHR. The pain threshold was assessed by the “Paw pressure” test, recognition short-term memory using a “Novel Object Recognition” test, and spatial memory was assessed using “T-maze alternation” test. The experiments are carried out at the end of peptide ICV infusion (day 13) and in the post-treatment period (month 3). Data are expressed as mean \pm SEM (n = 8). *p < 0.05 versus saline-treated control group; # p < 0.05 vs day 13 of infusion.

in pain regulation is still controversial [32]. SHRs have a deficit in recognition memory (normally about 80 %) (Fig. 4) and a small number of correct choices in the

T-maze. which correlates with poor spatial memory (Fig. 5). Long-term effects of NVK treatment were associated with improvement in spatial memory on the 3rd training day but did not change the recognition of the new object ($F_{1, 15} = 5.432, p = 0.03$; Fig. 5). The results from the memory tests showed that NVK treatment improved spatial memory but have not ameliorative action on the recognition short-term memory in female SHR. It is known that, on the one hand, pathological conditions of the cardiovascular system and peripheral nerves can up-regulate AT2 receptor density, and on the other hand, activation of brain AT2 receptors can lead to neuroprotection in various experimental brain injury models by bradykinin /B2 receptor/NO mechanism, improved cerebral blood flow, reduced oxidative stress and neuronal apoptosis [34 - 36]. Present data suggest a key role of brain AT2 receptors, as part of the protective RAS axis, in the regulation of more complex functions related to pain control, cognition, and memory in female SHR rats with disturbed behavioral characteristics.

CONCLUSIONS

In summary, chronic brain infusion with the hexapeptide NVK reduces anxiety, hyperactivity, increases pain threshold, and improves adaptation to the novel environment and spatial memory without changes in recognition memory in female SHR.

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