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EFFECTS OF NOVEL NOCICEPTIN ANALOGUES ON PAIN PERCEPTION DURING ACUTE AND CHRONIC IMMOBILIZATION STRESS

AUTHOR'S ABSTRACT

of the doctoral dissertation for the award of the educational and scientific degree "Doctor"

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CONTENT

LIST OF ABBREVIATIONS

ACTH – adrenocorticotropic hormone

Arg – arginine

ChIS – chronic immobilization stress

CRH – corticotropin-releasing hormone

HP – Hot Plate test

HPA – hypothalamic-pituitary-adrenal axis

i.p. – intraperitoneal administration

iNOS – inducible NO synthase

L-NAME – L-NG-Nitro-Arginine Methyl Ester

Lys – lysine

N/OFQ – nociceptin/orphanin

Nal – naloxone

NMDA – N-methyl-D-aspartate

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NOP – nociceptin receptor

NOS – nitric oxide synthase

Orn – ornithine

PAG – periaqueductal gray

POMC – proopiomelanocortin

PP – Paw Pressure test

PVN – paraventricular nucleus

SAM – sympathoadrenomedullary system

SIA – stress-induced analgesia

INTRODUCTION

The regulation of pain perception under stress conditions represents one of the most complex and relevant challenges in contemporary neurobiology and neuropharmacology. Stress is a multifaceted physiological and behavioral response of the organism to stimuli that threaten or disrupt homeostasis, activating a network of neural, endocrine, and immune mechanisms. In the short term, this response is adaptive and protective; however, prolonged exposure can lead to maladaptation, characterized by increased pain sensitivity, emotional instability, and impaired neuropeptide regulation.

One of the well-studied phenomena associated with the stress response is stress-induced analgesia (SIA) – a temporary reduction in pain sensitivity triggered by acute stressors such as immobilization, extreme temperatures, or social isolation. This reaction is mediated by multiple neuromodulatory systems, among which the endogenous opioid system plays a key role. Alongside it, significant scientific interest has been directed toward the nociceptin system, composed of the neuropeptide nociceptin/orphanin FQ (N/OFQ) and its specific receptor NOP.

N/OFQ is an endogenous neuropeptide with an anti-opioid profile. Although it is structurally similar to classical opioid peptides, it does not interact with MOR, DOR, or KOR receptors. Instead, it exerts its effects through selective activation of NOP – a G protein-coupled receptor characterized by a unique physiological profile. Activation of NOP suppresses neurotransmitter release and modulates pain, behavioral, emotional, and endocrine reactivity to stress.

Expressed in key brain structures such as the hypothalamus, amygdala, and the periaqueductal gray (PAG), NOP is involved in integrating the affective and sensory components of pain and in balancing adaptive and maladaptive responses during acute and chronic stress. The context-dependent activity of this system is particularly intriguing: while during acute stress N/OFQ may exert anxiolytic and antinociceptive effects, chronic stress leads to impaired receptor sensitivity, increased expression of prepronociceptin, and enhanced pain sensitivity. In view of these characteristics, the development of new synthetic analogues of N/OFQ emerges as a promising pharmacological approach. Of particular importance are the peptide modifications [Orn^{9}]N/OFQ(1–13)NH_2 and [Orn^{9}, Orn^{13}]N/OFQ(1–13)NH_2, in which the amino acid lysine is substituted with ornithine in key positions. These compounds demonstrate increased biostability and receptor selectivity, offering new possibilities for more precise modulation of pain under stress conditions.

The aim of the present dissertation is to investigate the effects of these new nociceptin analogues on pain perception in experimental models of acute and chronic immobilization stress, with a focus on their interaction with major neuromodulatory systems: nociceptinergic, opioid, and nitric oxide. The obtained results may contribute to a deeper understanding of the role of NOP-related

mechanisms in the regulation of pain and stress, as well as outline prospects for their application as pharmacological strategies in stress-associated pain conditions.

AIM AND TASKS

The aim of the present work is to investigate the analgesic effects of new nociceptin analogues – [Orn^9]N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 on pain perception, as well as their interaction with the opioid, nociceptin, and nitric oxide systems under acute and chronic immobilization stress.

To accomplish this aim, the following tasks were set:

1. To investigate the analgesic effects of N/OFQ(1-13)NH_2 and new nociceptin analogues – [Orn^9]N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 on nociception, using tests involving mechano- and thermoreceptors in:
    1.1. intact groups of animals;
    1.2. animals subjected to acute immobilization stress;
    1.3. animals subjected to chronic immobilization stress.

2. To investigate the influence of the NOP-selective antagonist JTC-801 on the analgesic effects of N/OFQ(1-13)NH_2 and the analogues – [Orn^9]N/OFQ(1-13)NH_2, [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 in:
    2.1. intact groups of animals;
    2.2. animals subjected to acute immobilization stress;
    2.3. animals subjected to chronic immobilization stress.

3. To investigate the influence of the non-selective opioid receptor antagonist naloxone on the analgesic effects of nociceptin N/OFQ(1-13)NH_2 and the analogues – [Orn^9]N/OFQ(1-13)NH_2, [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 in:
    3.1. intact groups of animals;
    3.2. animals subjected to acute immobilization stress;
    3.3. animals subjected to chronic immobilization stress.

4. To investigate the influence of the nitric oxide system on the analgesic effects of nociceptin N/OFQ(1-13)NH_2 and the analogues – [Orn^9]N/OFQ(1-13)NH_2, [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2, through L-NAME (an inhibitor of nitric oxide synthase, NOS) and L-arginine (a precursor of NO) in:
    4.1. intact groups of animals;
    4.2. animals subjected to acute immobilization stress;
    4.3. animals subjected to chronic immobilization stress.

5. To investigate the effects of nociceptin N/OFQ(1-13)NH_2 and the analogues [Orn^9]N/OFQ(1-13)NH_2, [Orn^9,Orn^{13}]N/OFQ(1-13)NH_2 on the serum levels of ACTH, cortisol, and adrenaline under acute and chronic immobilization stress.

MATERIALS AND METHODS

The experiments were conducted on 402 male Wistar rats (180–200 g), divided into groups of 6 and housed in polypropylene cages under normal conditions, with free access to water and food, at a temperature of 22 ± 2°C. Light was regulated in a 12-hour light/12-hour dark cycle. The experiments were conducted between 9:00 and 12:00 h.

The study was carried out in accordance with the national and international requirements for the protection and humane treatment of laboratory animals (European Directive 2010/63/EU) and was approved by the Bulgarian Food Safety Agency (permit registration № 239/16.09.2019).

I. In vivo methods

1. Nociceptive tests

1.1. Method with application of mechanical stimulation – paw pressure (PP) test – Randall–Selitto test

To assess mechanical pain sensitivity in experimental animals, a method involving mechanical stimulation was used – the Paw Pressure test, also known as the Randall–Selitto test. This is a classical in vivo method, sensitive to peripherally acting analgesics, which allows quantitative measurement of the pain threshold in laboratory animals.

In the present study, an analgesimeter (Ugo Basile, Italy) was used, through which a gradually increasing mechanical pressure was applied to the rat's hind paw, placed under a rounded metal tip with a diameter of approximately 1 mm. The animal was manually stabilized so as to minimize additional stress and ensure the accuracy of the measurement.

The measurement of the pain threshold was performed by recording a characteristic motor reaction – withdrawal of the paw, occurring upon reaching the nociceptive threshold. The pressure was graded and recorded in relative units according to the scale of the apparatus, with the maximum applicable force limited to 500 g to prevent tissue damage. The test also allows the registration of

supraspinal reactions (vocalization), which may serve as an additional indicator of pain perception.

Figure 1. Method with application of mechanical stimulation – Paw pressure test

1.2. Method with application of thermal stimulation – Hot Plate (HP) test

To assess thermal pain sensitivity, the HP test was applied, which is an established behavioral model for studying central nociceptive activity. The test was conducted on freely moving laboratory rats, with a thermal stimulus at a temperature of 55 ± 1°C applied to the plantar surface of the paws. Measurement of the pain threshold was performed by recording the latency time – the interval from the beginning of the thermal stimulation to the appearance of a characteristic reaction of the animal (paw licking, jumping), indicating the perception of pain. The reaction time was recorded in seconds, with longer latency interpreted as an elevated pain threshold or the presence of analgesia.

To prevent tissue damage, a maximum exposure time (cut-off time), usually 30 seconds, was applied. The test was performed in a controlled environment with minimal external stimuli, and the animals were previously acclimated to the experimental setting. The measurements were carried out in several consecutive sessions, allowing evaluation of the dynamics of the nociceptive threshold over time and under the influence of experimental interventions.

Figure 2. Method with application of thermal stimulation – Hot plate test

2. Methods for induction of stress

In the present study, two established models of acute and chronic immobilization stress were used to induce psychophysiological stress in laboratory rats.

2.1. Acute immobilization stress

In acute immobilization stress, the animals were placed once for 1 hour in transparent plastic cylinders with ventilation openings, which restricted their movements without causing physical injury.

2.2. Chronic immobilization stress

In the chronic immobilization stress protocol, animals were placed daily for 3 hours in the same cylinders over a period of 4 consecutive days. This protocol was adapted from models used to induce depression-like behavior and behavioral changes associated with prolonged stress exposure. In both models, individual cylinders were used, adjusted to the size of each animal, with sufficient openings for breathing and no possibility of movement, while adhering to ethical standards to minimize discomfort. Animals were acclimated to the experimental environment before the start of the procedures, and control groups were kept under identical conditions without stress exposure.

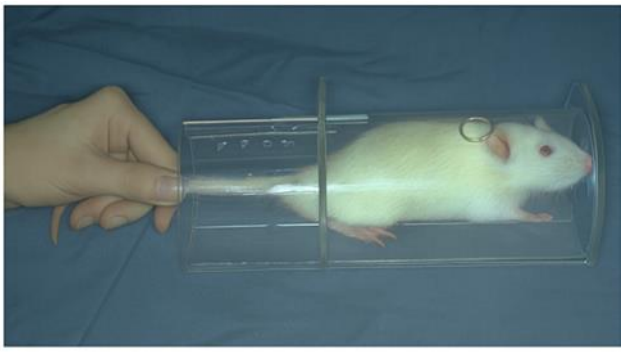

Figure 3. Immobilization stress model

II. Immunological Methods

For the quantitative determination of adrenocorticotropic hormone (ACTH), cortisol, and adrenaline concentrations in rat serum samples, an ELISA (Enzyme-Linked Immunosorbent Assay) was used. ACTH concentration was measured using a rat-specific ELISA kit (MyBioSource, San Diego, CA, USA), and the results were reported in pg/ml. Cortisol levels were determined using a similar ELISA kit from the same manufacturer, with values reported in ng/ml. Adrenaline (epinephrine) concentration was also measured using an ELISA kit (MyBioSource, San Diego, CA, USA) and expressed in ng/ml. The procedure was performed according to the manufacturer's instructions, ensuring high sensitivity and specificity for each of the analyzed compounds.

III. Synthesis of nociceptin N/OFQ(1-13)NH_2 analogues

The nociceptin N/OFQ(1-13)NH_2 analogues – [Orn^9]N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 were synthesized by the team of Prof. Dr. Eng. Emilia Naydenova, DSc, from the Department of Organic Chemistry, Faculty of Chemical Technology, University of Chemical Technology and Metallurgy – Sofia.

The synthesis was performed using the solid-phase peptide synthesis (SPPS) method, employing the Fmoc strategy. In these modified analogues, the amino acid lysine (Lys) at positions 9 and/or 13 was replaced with ornithine (Orn) to investigate the effect of this structural modification on the peptide's analgesic activity and its interaction with the opioid, nociceptin, and nitric oxide systems under acute and chronic immobilization stress.

The following novel fragment analogues of N/OFQ(1-13)NH_2 were obtained:

- [Orn^9]N/OFQ(1-13)NH_2: H-Phe^1-Gly^2-Gly^3-Phe^4-Thr^5-Gly^6-Ala^7-Arg^8-Orn^9-Ser^{10}-Ala^{11}-Arg^{12}-Lys^{13}-NH_2
- [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2: H-Phe^1-Gly^2-Gly^3-Phe^4-Thr^5-Gly^6-Ala^7-Arg^8-Orn^9-Ser^{10}-Ala^{11}-Arg^{12}-Orn^{13}-NH_2

Experimental Protocols

For nociceptive methods: Animals were divided into groups of six. All substances were dissolved in 0.9% NaCl solution and administered intraperitoneally (i.p.). N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] – all at a dose of 10 μg/kg, were administered as follows:

1. In intact animals;
2. In intact animals 10 min after JTC-801 (N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide monohydrochloride) at a dose of 0.5 mg/kg;
3. In intact animals 20 min after Naloxone hydrochloride dihydrate (Nal, 1 mg/kg), L-NG-Nitro-Arginine Methyl Ester (L-NAME, 10 mg/kg), or L-arginine (L-arg, 1 mg/kg);
4. Immediately after the stress procedure;
5. After stress and 10 min following JTC-801 (0.5 mg/kg);
6. After stress and 20 min following Naloxone hydrochloride dihydrate (1 mg/kg), L-NAME (10 mg/kg), or L-arginine (1 mg/kg).

All substances were purchased from Sigma-Aldrich (Merck, Germany).

The control group consisted of intact animals injected with saline solution (1 ml/kg). Nociceptive measurements were started immediately after stress or 10 min after peptide administration.

For the ELISA analysis: Animals were divided into 9 groups (n = 6). The control group consisted of intact animals. Two of the groups were subjected only to one of the two stress models – acute immobilization stress (1hIS) or chronic immobilization stress (CIS) – and were injected only with saline solution. The remaining groups were exposed to one of the two types of stress (1hIS or CIS) and injected with nociceptin or one of the two analogues (all at a dose of 10 μg/kg, i.p.).

For the ELISA analysis to determine serum levels of ACTH, cortisol, and adrenaline, a single blood sample was collected via puncture of the sublingual

vein. Animals were anesthetized with ketamine (90 mg/kg, i.p.). The sublingual vein puncture was performed using a sterile needle (27–30G), collecting approximately 1 ml of blood in a tube without anticoagulant. Samples were allowed to clot at room temperature for 30–45 minutes, then centrifuged at 3000 rpm for 10–15 minutes. The resulting serum was transferred to clean tubes and stored at –20°C. After thawing, serum samples were used for quantitative determination of hormone levels using competitive ELISA methods according to the instructions of the respective kits (ACTH – MBS2700345, cortisol – MBS701698, adrenaline – MBS732836, MyBioSource, San Diego, CA, USA).

IV. Statistical analysis

Quantitative variables were described by calculating the mean and standard error of the mean (SEM) to characterize the central tendency and variability of the data. To test the hypothesis of differences between groups, a one-way analysis of variance (ANOVA) was applied. In cases where statistically significant differences were found, a Bonferroni post hoc test was used to perform multiple comparisons between groups. The threshold of statistical significance was set at $p<0.05$.

To clearly illustrate differences between the experimental groups, the results were visualized using bar graphs showing mean values, 95% confidence intervals (95% CI), and statistical significance (p-value).

Statistical data analysis was performed using IBM SPSS Statistics software, version 26, and Microsoft Excel 2019 was used for graphical representation of the results. The obtained results are presented in detail in the “Results” section.

RESULTS AND DISCUSSION

1. Analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on nociception using mechanical (PP) and thermal (HP) tests:

1.1. Intact animal groups

Paw Pressure Test

In the PP test, N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] significantly increased the pain threshold at 10 and 20 minutes compared to the control group ($p<0.001$). The highest pain threshold values were observed at 10 minutes: [Orn^9, Orn^{13}] (318 g/cm² ± 3.742), [Orn^9] and N/OFQ(1-13)NH_2 (306

g/cm² ± 2.449 and 306 g/cm² ± 8.124, respectively). At 20 minutes, all peptides showed a statistically significant difference compared to the control ($p<0.001$): N/OFQ(1-13)NH_2 (210 g/cm² ± 5.477), [Orn^9] (226 g/cm² ± 5.099), and [Orn^9, Orn^{13}] (218 g/cm² ± 3.742).
By 30 minutes, a decrease in the pain threshold was observed for all peptides, with values comparable to those of the control group, indicating a fading of the analgesic effect (fig. 4).

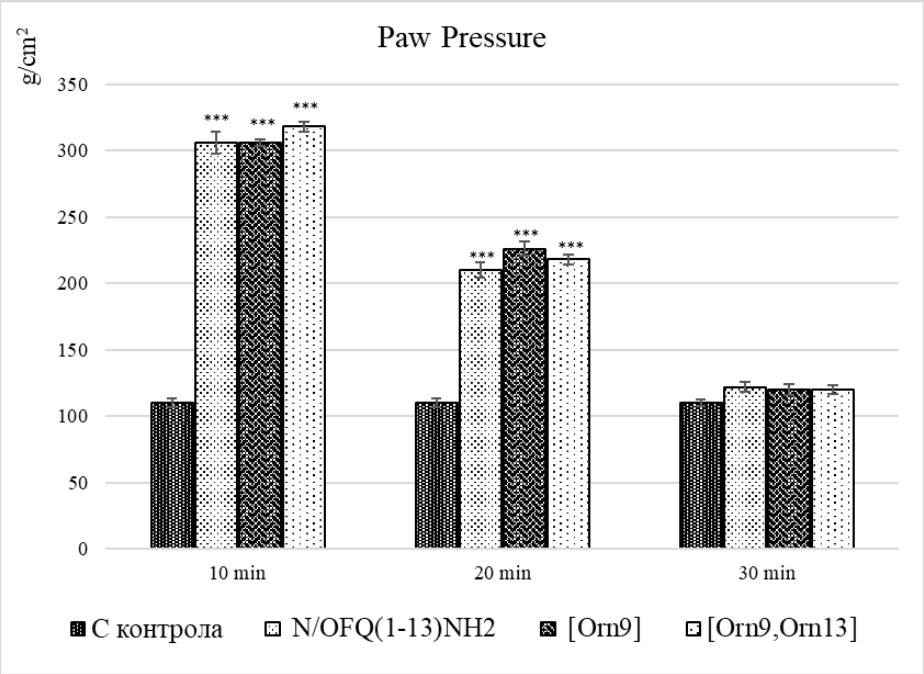

*Figure 4. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}], all at a dose of 10 µg/kg, i.p., on the pain threshold in intact animals. Data are presented as mean ± S.E.M.; ***p<0.001 vs. control.*

Hot Plate Test

At 10 minutes after administration, a statistically significant prolongation of latency time was observed in all experimental groups compared to the control ($p<0.001$). The recorded values were as follows:

- N/OFQ(1-13)NH_2: 8.4 ± 0.24 s
- [Orn^9]: 9.1 ± 0.15 s
- [Orn^9,Orn^{13}]: 9.56 ± 0.12 s

At 20 minutes, N/OFQ(1-13)NH_2 significantly increased latency time compared to the control group ($p<0.05$). [Orn^9] and [Orn^9, Orn^{13}] also showed increased latency, but the difference was not statistically significant.

At 30 minutes, a statistically significant decrease in latency time was observed compared to the control group ($p<0.05$) for N/OFQ(1-13)NH_2 and ($p<0.001$) for [Orn^9, Orn^{13}]. For [Orn^9], latency time was comparable to that of the control group (fig. 5).

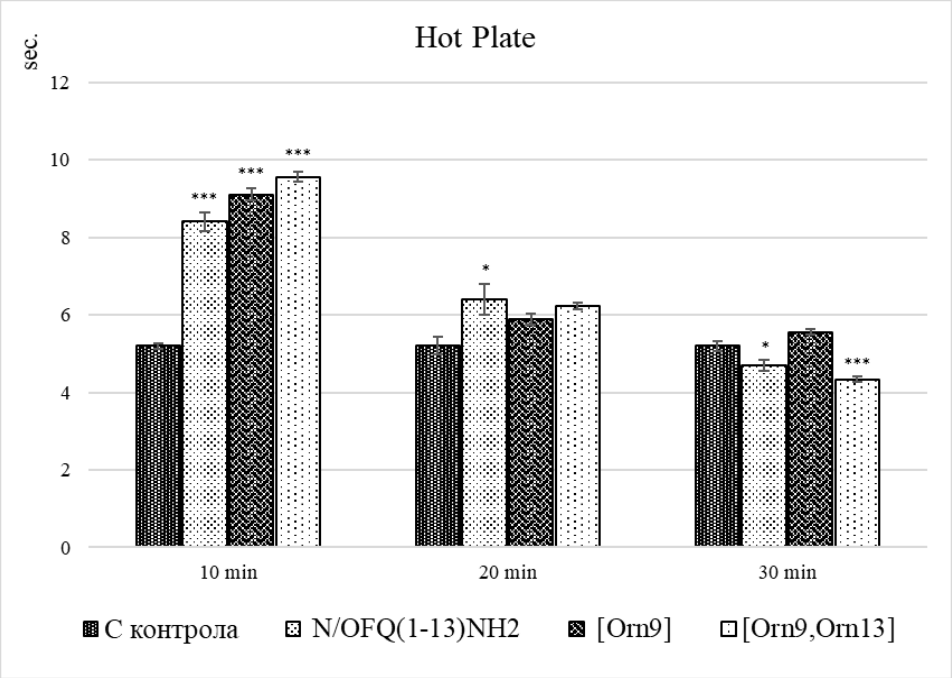

*Figure 5. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on HP latency in intact animals. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 vs. control.*

1.2. Analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on nociception using mechanical (PP) and thermal (HP) tests in animals subjected to acute immobilization stress

Paw Pressure test

Results from the PP test showed that the group subjected to acute immobilization stress (1hIS) had a statistically significant increase in pain threshold compared to the control group throughout the study period ($p<0.001$).
Following stress, N/OFQ(1-13)NH_2 and its analogues significantly reduced the pain threshold compared to the 1hIS group ($p<0.001$) over the entire study period. Compared to the control group, a significant increase in pain threshold was still observed ($p<0.001$) (fig. 6).

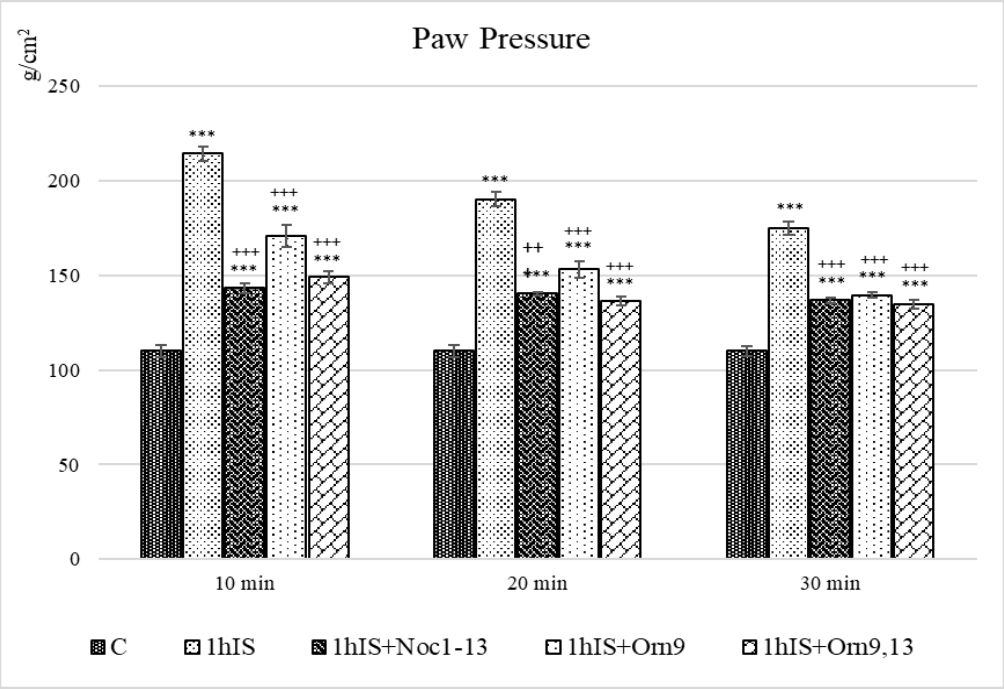

*Figure 6. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on pain threshold following 1hIS. Data are presented as mean ± S.E.M.; ***p<0.01 vs. control; $^{+++}$p<0.001 vs. 1hIS.*

Hot Plate Test

The group of animals subjected to 1hIS showed a statistically significant prolongation of latency time at 10 minutes compared to the control group ($p<0.01$).

Administration of N/OFQ(1-13)NH_2 after stress resulted in a statistically significant shortening of latency time at 10 minutes compared to 1hIS ($p<0.001$).
The analogues [Orn^9] and [Orn^9, Orn^{13}] following stress significantly shortened latency time compared to 1hIS throughout the study period ($p<0.01$; $p<0.001$).
For [Orn^9, Orn^{13}], a statistically significant decrease in latency time compared to the control group was observed ($p<0.05$ at 10 min; $p<0.01$ at 20 and 30 min), while for [Orn^9] this effect was significant at 30 minutes ($p<0.05$) (fig. 7).

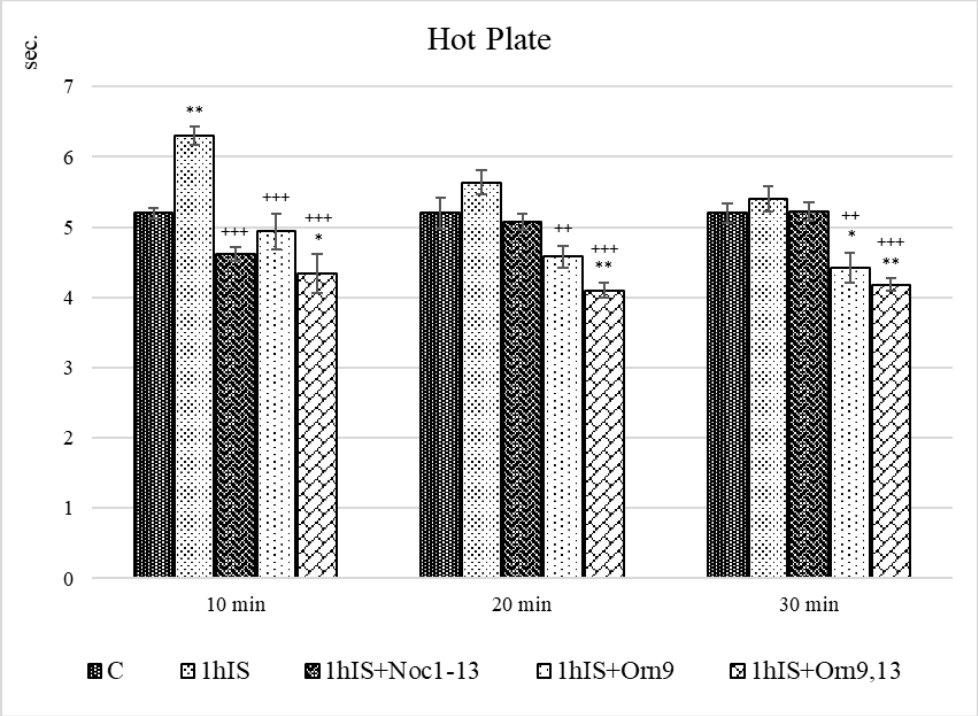

*Figure 7. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on HP latency following 1hIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01 vs. control; $^{++}$p<0.01; $^{+++}$p<0.001 vs. 1hIS.*

1.3. Analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on nociception using mechanical (PP) and thermal (HP) tests in animals subjected to chronic immobilization stress (ChIS)

Paw Pressure Test

The results showed that animals subjected to ChIS had a statistically significant increase in pain threshold compared to the control group at 10 minutes ($p<0.05$).

Following stress, N/OFQ(1-13)NH_2 decreased the pain threshold at 10 and 30 minutes, but the difference compared to ChIS was not statistically significant, while at 20 minutes the response was comparable to that of the ChIS animals.

The analogue [Orn^9] significantly decreased the pain threshold at 30 minutes compared to ChIS ($p<0.05$), whereas [Orn^9, Orn^{13}] reduced the pain threshold throughout the entire study period: at 10 minutes ($p<0.001$), at 20 minutes ($p<0.05$), and at 30 minutes ($p<0.001$) compared to ChIS, and also compared to the control group ($p<0.05$ at 10 min; $p<0.001$ at 30 min) (fig. 8).

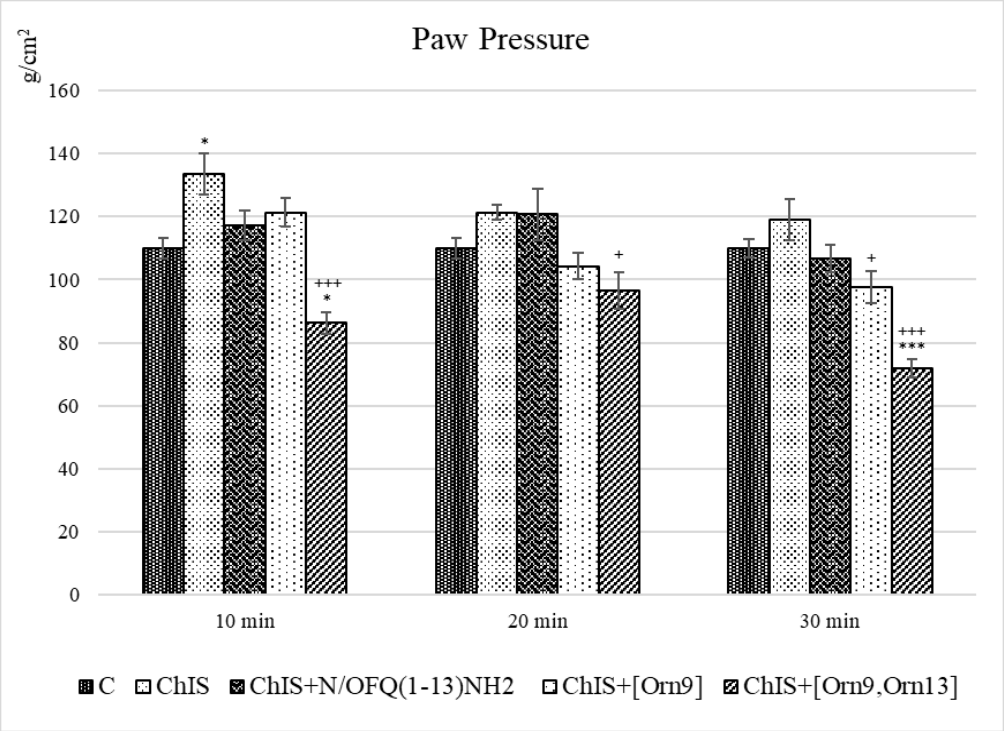

*Figure 8. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on pain threshold following ChIS. Data are presented as mean ± S.E.M.; ***p < 0.001 vs. control; $^{+}p<0.05$; $^{+++}p<0.001$ vs. ChIS.*

Hot Plate test

The results show that ChIS significantly prolonged latency time compared to the control group at 10 minutes ($p<0.01$). At 20 minutes, latency remained prolonged, and at 30 minutes it was comparable to the control group.

Administration of N/OFQ(1-13)NH_2 and its analogues immediately after stress resulted in a statistically significant shortening of latency time at 10 and 20 minutes compared to the ChIS group:

- ChIS + N/OFQ(1-13)NH_2: 10 min ($p<0.01$)
- [Orn^9]: 10 min ($p<0.001$) and 20 min ($p<0.05$)
- [Orn^9,Orn^{13}]: 10 min and 20 min ($p<0.01$) (fig. 9).

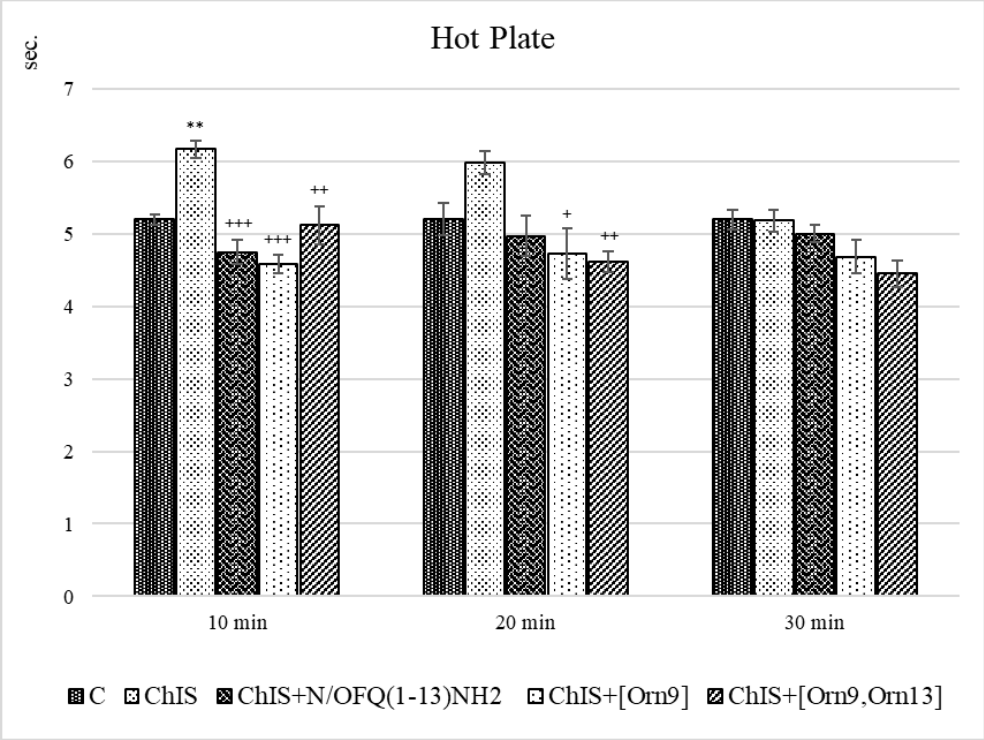

*Figure 9. Effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 μg/kg, i.p.) on HP latency following ChIS. Data are presented as mean ± S.E.M.; **p<0.01 vs. control, $^{+}$p<0.05; $^{++}$p<0.01; $^{+++}$p<0.001 vs. ChIS.*

1.4. Discussion

In the present study, the analgesic effects of N/OFQ(1-13)NH_2 and its structural analogues – [Orn^9] and [Orn^9, Orn^{13}] were evaluated in intact animals and in animals subjected to acute and chronic immobilization stress. The peptides were administered i.p. at a dose of 10 μg/kg, and pain sensitivity was assessed using two tests: PP for mechanical nociception and HP for thermal nociception.

Results from the PP test showed that all peptides significantly increased the pain threshold at 10 and 20 minutes ($p<0.001$), indicating a pronounced analgesic effect under mechanical stimulation. The highest values were observed at 10 minutes, with [Orn^9, Orn^{13}] showing the strongest effect. This suggests that the dual ornithine modification may enhance interaction with the NOP receptor and improve peptide stability.

No statistically significant differences were observed among the peptides themselves ($p>0.05$), indicating that their analgesic activity is comparable in strength and duration within the test. At 30 minutes, the effect diminished, with

values approaching those of the control group (fig. 4), consistent with the short-lived nature of analgesia reported for NOP agonists.

In the HP test, a statistically significant prolongation of latency time was observed at 10 minutes for all peptides ($p<0.001$), confirming their analgesic effect under thermal stimulation. At 20 minutes, the effect decreased, with only N/OFQ(1-13)NH_2 maintaining a statistically significant difference ($p<0.05$) compared to the control. At 30 minutes, a statistically significant shortening of latency was observed ($p<0.05$) for N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}], while for [Orn^9] the values were comparable to the control group (fig. 5).

These results correspond to the described dual role of the N/OFQ–NOP system, which can exhibit both antinociceptive and pronociceptive effects depending on dose, localization, and type of pain stimulus. The hyperalgesia observed at 30 minutes is likely due to receptor desensitization or compensatory activation of pronociceptive mechanisms.

Differences in the results between the PP and HP tests can be explained by their distinct physiological bases. The PP test assesses responses to mechanical stimuli and is more sensitive to peripheral mechanisms of analgesia. The analgesic effect observed in this test is likely due to peripheral activation of NOP receptors, which inhibits the release of mediators such as substance P, glutamate, and GABA from presynaptic terminals. This reduces the excitability of nociceptive pathways and increases the pain threshold. In contrast, the HP test measures responses to thermal stimuli and predominantly reflects central integration of pain signals. The effects observed in this test are likely mediated by central structures such as the PAG and the thalamus, which participate in integration and modulation of nociceptive information through descending inhibitory mechanisms.

This differential sensitivity suggests that the observed peptide effects are mediated at different levels of the nociceptive system depending on the type of experimental model used. Additionally, structural modifications of the analogs at positions 9 and 13 may influence receptor selectivity, stability, and duration of effect. This could explain the differences in the temporal dynamics of analgesia and the occurrence of hyperalgesia for N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}] in the HP test.

Stress represents a complex physiological and behavioral response of the organism to external or internal stimuli, involving activation of the HPA axis, the autonomic nervous system, and multiple neurotransmitter systems. One well-documented phenomenon associated with acute stress is stress-induced analgesia (SIA) – a temporary reduction in pain sensitivity, considered an adaptive mechanism that supports survival under conditions of threat or high demand. Results from the PP test showed a statistically significant increase in pain threshold in animals subjected to acute stress compared to the control group ($p<0.001$). At 10

minutes, the threshold increased by 94.8%, at 20 minutes by 73.0%, and at 30 minutes by 59.1% compared to controls (fig. 6). These results confirm the presence of SIA and are consistent with literature data indicating that acute stress activates endogenous antinociceptive mechanisms. In the HP test, a statistically significant prolongation of latency was observed at 10 minutes compared to the control group ($p<0.01$), further confirming SIA (fig. 7). The gradual shortening of latency at 20 and 30 minutes in the 1hIS group indicates that the SIA effect is transient. In the PP test, nociceptin and its analogues administered after 1hIS significantly decreased the pain threshold compared to the stress-only group ($p<0.001$) and significantly increased it compared to the control group ($p<0.001$) throughout the study period, indicating that the peptides suppress SIA, with [Orn^9] showing a weaker effect at 10 minutes (fig. 6). These results support literature data suggesting that NOP agonists modulate, but do not completely abolish, endogenous analgesia.

In the HP test, peptide administration following stress led to a statistically significant shortening of latency compared to the 1hIS group ($p<0.001$) at 10 minutes, as well as compared to the control group ($p<0.05$) for [Orn^9,Orn^{13}]. At 20 and 30 minutes, a significant shortening of latency was observed compared to 1hIS ($p<0.01$ for [Orn^9], $p<0.001$ for [Orn^9, Orn^{13}]), as well as compared to the control group ($p<0.01$ for [Orn^9, Orn^{13}] at 20 and 30 minutes; $p<0.05$ for [Orn^9] at 30 minutes) (fig. 7). These HP test results confirm that nociceptin and its analogues suppress SIA. The observed hyperalgesia indicates pronociceptive activity of the peptides, particularly pronounced for [Orn^9] and [Orn^9, Orn^{13}].

In a comparative analysis of the PP and HP tests, it was observed that the pronociceptive effect of the peptides is more pronounced under thermal stimulation. [Orn^9,Orn^{13}] significantly shortened the latency compared to both the 1hIS and control groups throughout the study period (figs. 6 and 7). These effects are consistent with the concept of modality-selective SIA and highlight the interplay between stress, pain, and neuropeptide regulation.

Results from the PP test showed that animals subjected to ChIS significantly increased their pain threshold at 10 minutes compared to the control group ($p<0.05$), confirming the presence of SIA described in chronic stress models (fig. 8). Similar effects were observed in the HP test, where ChIS significantly prolonged latency at 10 minutes ($p<0.01$), confirming the antinociceptive effect of stress (fig. 9).

Administration of N/OFQ(1-13)NH_2 after stress led to a reduction in pain threshold compared to ChIS at 10 and 30 minutes, indicating that N/OFQ(1-13)NH_2 suppresses SIA. In the HP test, N/OFQ(1-13)NH_2 significantly shortened latency compared to ChIS at 10 minutes ($p<0.01$). Compared to the control group, latency was relatively shorter, suggesting that the peptide completely abolishes SIA while inducing hyperalgesia (figs. 8 and 9).

The analogue [Orn^9] significantly decreased the pain threshold at 30 minutes compared to ChIS ($p<0.05$), thereby suppressing stress-induced analgesia. At 20 and 30 minutes in the PP test, the pain threshold was relatively lower than in the control group, indicating a pronociceptive effect of the peptide. In the HP test, [Orn^9] significantly shortened latency at 10 minutes ($p<0.001$) and 20 minutes ($p<0.05$) compared to ChIS. Latency values for [Orn^9] were relatively lower than the control group, confirming the pronociceptive effect of the analogue under chronic stress (figs. 8 and 9).

The strongest pronociceptive effect in the PP test was observed for [Orn^9, Orn^{13}], which significantly decreased the pain threshold compared to ChIS throughout the study period. Significant differences in pain threshold were also observed compared to the control group at 10 minutes ($p<0.05$) and 30 minutes ($p<0.001$). In the HP test, [Orn^9, Orn^{13}] significantly shortened latency at 10 and 20 minutes ($p<0.01$) compared to the ChIS group (figs. 8 and 9).

The results indicate that nociceptin and its analogues suppress analgesia induced by ChIS, with the pronociceptive effect being more pronounced for [Orn^9] and [Orn^9, Orn^{13}]. Chronic stress may induce desensitization of NOP receptors, leading to reduced sensitivity to nociceptin ligands, likely through changes in receptor expression or conformational state and via increased activity of antagonistic neurotransmitter systems such as CRH and glutamate. Consequently, the N/OFQ–NOP system may modulate or even reverse the effects of endogenous analgesia, resulting in dynamic regulation of pain sensitivity depending on the neurochemical environment. Under chronic stress, impaired regulation of NOP receptors and their interaction with other neuromodulatory systems may explain the pronociceptive effects of the peptides and the observed hyperalgesia in response to both mechanical and thermal stimuli.

2. Effect of the NOP-selective antagonist JTC-801 on the analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}]:

2.1. Intact animal groups

Paw Pressure test

Administration of JTC-801 alone significantly increased the pain threshold throughout the study period compared to the control group ($p<0.001$). Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9,Orn^{13}] led to a significant reduction in the pain threshold compared to JTC-801 alone for the entire study period ($p<0.01$; $p<0.001$). At 10 minutes, [Orn^9] and [Orn^9, Orn^{13}] significantly decreased the pain threshold compared to JTC-801

($p<0.01$). For N/OFQ(1-13)NH_2, the pain threshold was also reduced, although not significantly compared to JTC-801. At 20 and 30 minutes, nociceptin and its analogues significantly reduced the pain threshold compared to JTC-801 ($p<0.01$; $p<0.001$). Compared to the control group, all peptides showed a significantly higher pain threshold throughout the study period ($p<0.001$) (fig. 10).

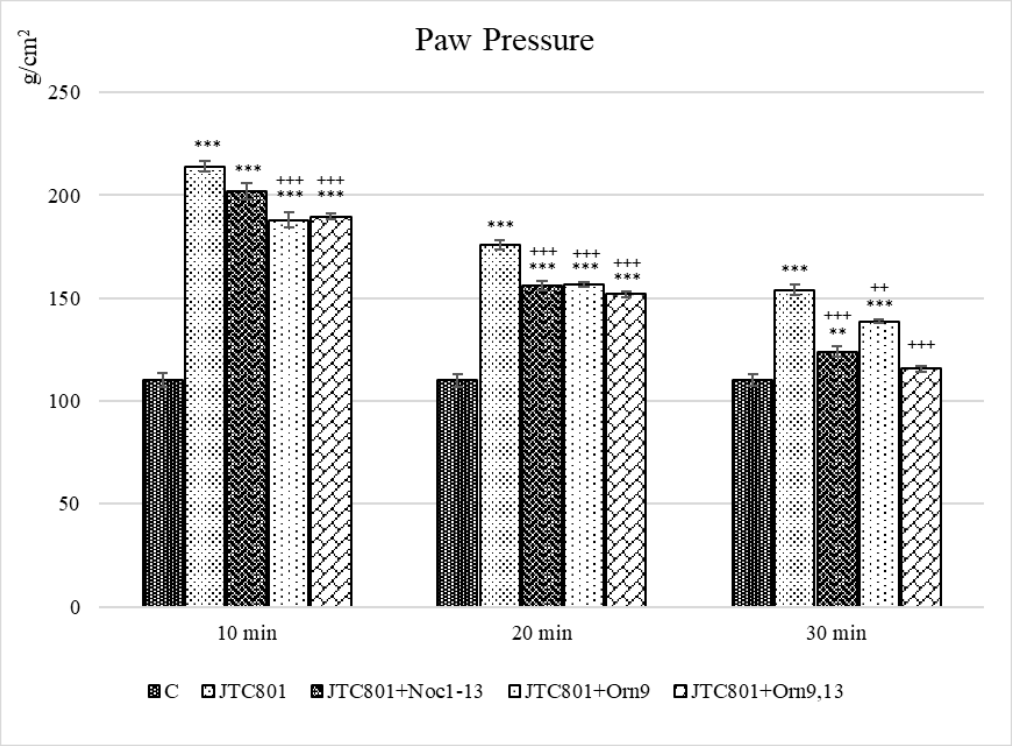

*Figure 10. Effects of JTC-801 in combination with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on pain threshold in intact animals. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 vs. control; $^{+}$p<0.05; $^{++}$p<0.01; $^{+++}$p<0.001 vs. JTC-801.*

Hot Plate test

Administration of JTC-801 alone significantly prolonged latency compared to control at 10, 20, and 30 minutes ($p<0.001$, 9.6 sec; $p<0.001$, 6.7 sec; $p<0.01$, 5.8 sec, respectively).

Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and its analogues [Orn^9] and [Orn^9, Orn^{13}] led to a significant shortening of latency compared to JTC-801 alone throughout the entire observation period ($p<0.001$, $p<0.01$).

For [Orn^9] and [Orn^9, Orn^{13}], latency was significantly reduced compared to the control group at 10 and 20 minutes ($p<0.01$) and at 30 minutes ($p<0.001$). N/OFQ(1-13)NH_2 significantly shortened latency at 30 minutes ($p<0.05$) (fig. 11).

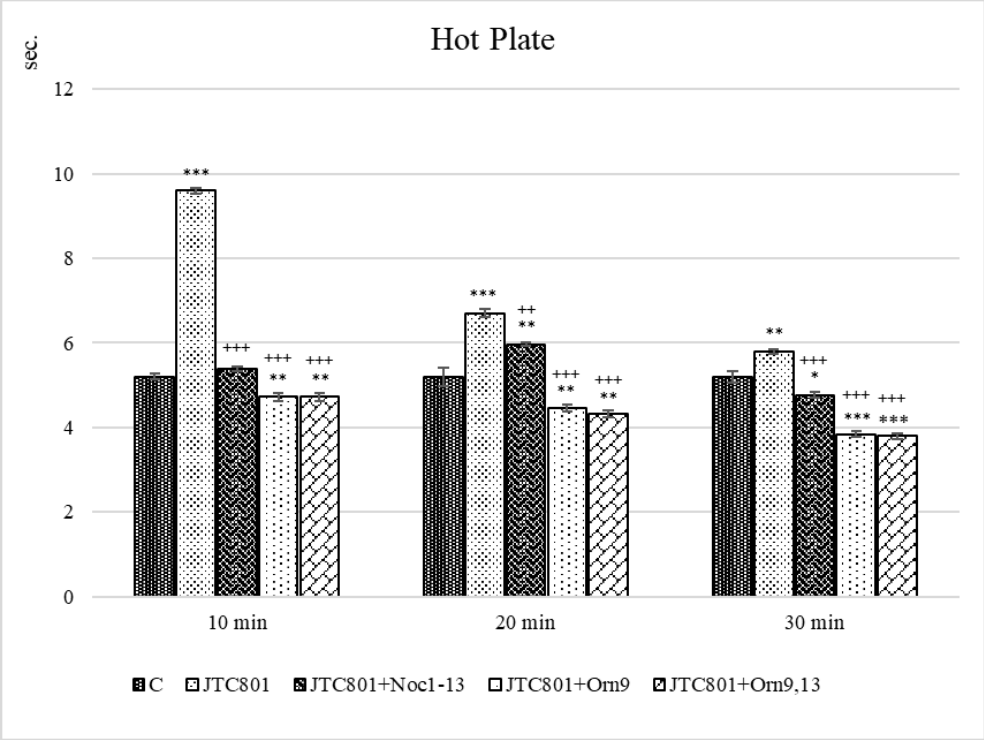

*Figure 11. Effects of JTC-801 in combination with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on HP latency in intact animals. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 vs. control; $^{+}$p<0.05; $^{++}$p<0.01; $^{+++}$p<0.001 vs. JTC-801.*

2.2. Effect of the NOP-selective antagonist JTC-801 on the analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9,Orn^{13}] in animals subjected to 1h immobilization stress (1hIS)

Paw Pressure test

The 1hIS group showed a statistically significant increase in pain threshold compared to the control group (p<0.001) throughout the observation period: 10 min (214.33 g/cm²/110), 20 min (190.33 g/cm²/110), and 30 min (175 g/cm²/110). Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and its analogues after stress resulted in a significant decrease in pain threshold compared to 1hIS (p<0.01; p<0.001), while still showing a significant increase compared to the control group (p<0.001) for the entire observation period (fig. 12).

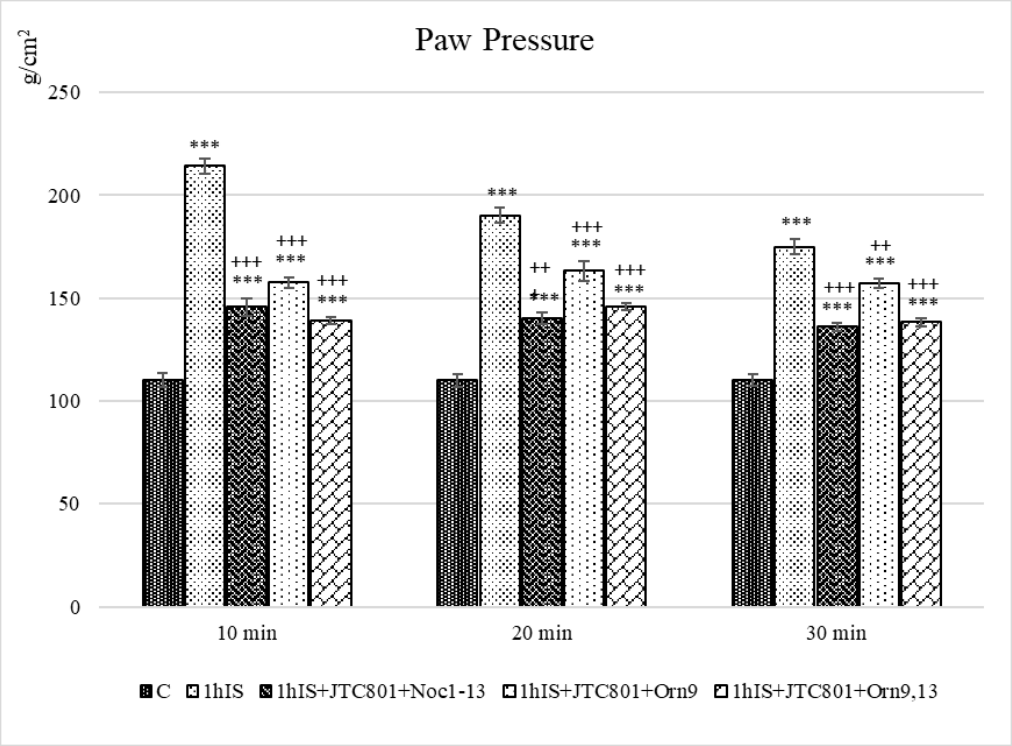

*Figure 12. Effects of co-administration of JTC-801 (0.5 mg/kg, i.p.) with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on pain threshold after 1hIS. Data are presented as mean ± S.E.M.; ***p<0.001 vs. control; $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ vs. 1hIS.*

Hot Plate test

The 1hIS group showed a statistically significant increase in latency compared to the control group only at 10 min ($p<0.05$).
Co-administration of JTC-801 with nociceptin and its analogues after stress resulted in a significant decrease in latency at 10 min: [Orn^9] ($p<0.001$) and [Orn^9, Orn^{13}] ($p<0.05$) compared to 1hIS.

At 20 and 30 min, no statistically significant differences were observed between the groups (fig. 13).

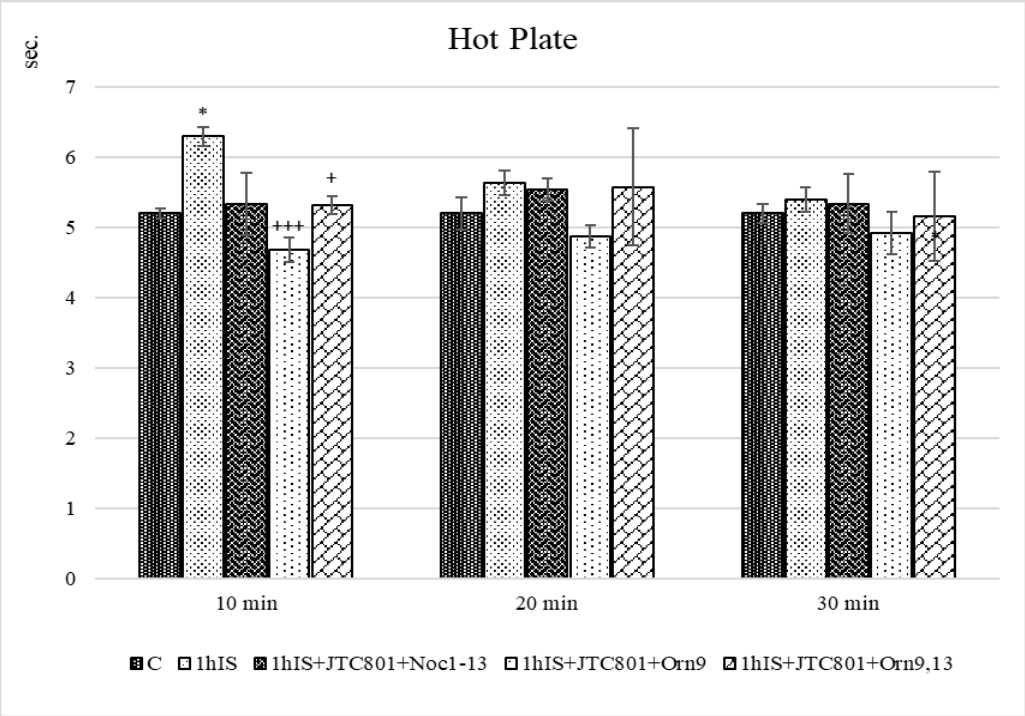

*Figure 13. Effects of co-administration of JTC-801 (0.5 mg/kg, i.p.) with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on HP latency after 1hIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 vs. control; $^{+}$p<0.05; $^{++}$p<0.01; $^{+++}$p<0.001 vs. 1hIS.*

2.3. Effects of the NOP-selective antagonist JTC-801 on the analgesic activity of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] in animals subjected to ChIS

Paw Pressure test

Chronic immobilization stress (ChIS) led to a statistically significant increase in pain threshold at 10 min ($p<0.001$) and 20 min ($p<0.05$) compared to the control group.

Administration of JTC-801 alone immediately after ChIS significantly decreased the pain threshold compared to both ChIS and control groups throughout the observation period ($p<0.001$).

At 10 min, the groups JTC-801 + N/OFQ(1-13)NH_2, JTC-801 + [Orn^9], and JTC-801 + [Orn^9, Orn^{13}] showed a statistically significant decrease in pain threshold compared to ChIS ($p<0.001$). The JTC-801 + N/OFQ(1-13)NH_2 group also showed a significant decrease compared to the control group ($p<0.05$).

At 20 min, a significant decrease in pain threshold was observed in the JTC-801 + N/OFQ(1-13)NH_2 and JTC-801 + [Orn^9, Orn^{13}] groups compared to ChIS ($p<0.01$).

At 30 min, only the [Orn^9, Orn^{13}] group showed a significant decrease in pain threshold compared to ChIS ($p<0.05$), while the other groups did not show significant differences (fig. 14).

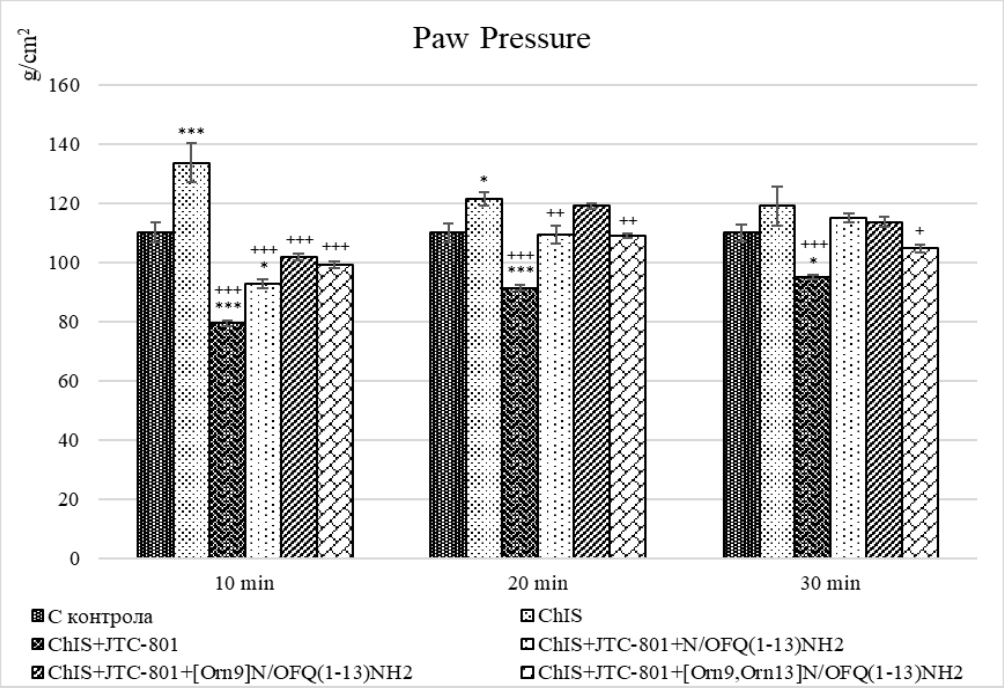

*Figure 14. Effects of co-administration of JTC-801 (0.5 mg/kg, i.p.) with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 μg/kg, i.p.) on pain threshold after ChIS. Data are presented as mean ± S.E.M.; *$p<0.05$; ***$p<0.001$ vs. control; $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ vs. ChIS.*

Hot Plate test

Chronic immobilization stress (ChIS) significantly increased latency compared to control at 10 min ($p<0.001$) and 20 min ($p<0.01$).

Administration of JTC-801 immediately after chronic stress significantly decreased latency compared to both ChIS and control groups throughout the observation period ($p<0.001$). Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and the analogues [Orn^9] and [Orn^9, Orn^{13}] after stress also significantly decreased latency compared to ChIS and control ($p<0.001$) throughout the observation period (fig. 15).

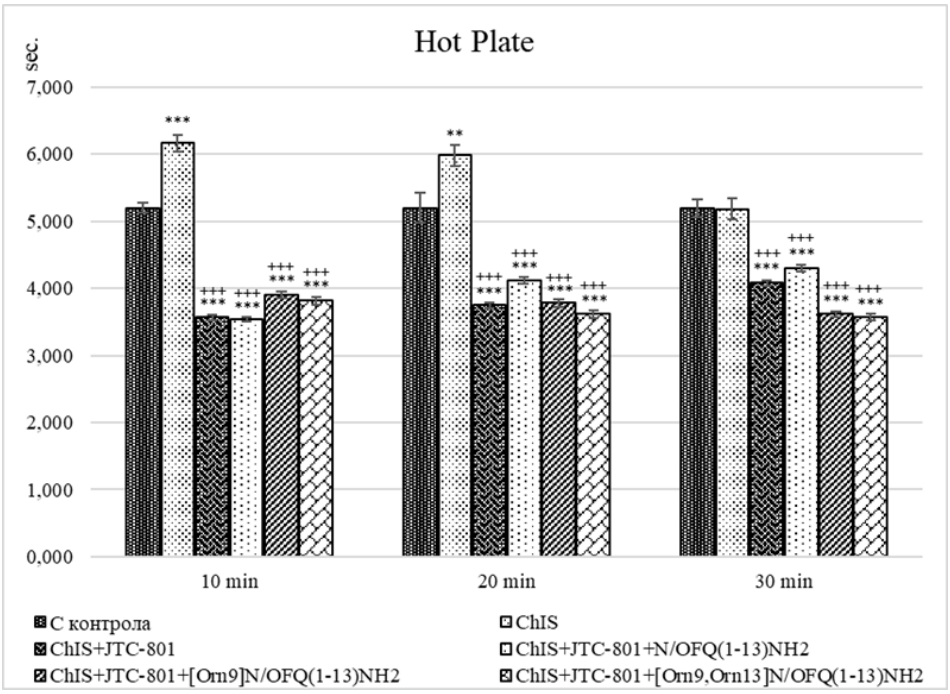

*Figure 15. Effects of co-administration of JTC-801 (0.5 mg/kg, i.p.) with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 μg/kg, i.p.) on HP latency after ChIS. Data are presented as mean ± S.E.M.; **p<0.01; ***p<0.001 vs. control; $^{+++}$p<0.001 vs. ChIS.*

2.4. Discussion

The present study aimed to evaluate the influence of the NOP-selective antagonist JTC-801 on the analgesic effects of N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] in intact animals and in animals subjected to acute and chronic immobilization stress.

Results from the Paw Pressure (PP) and Hot Plate (HP) tests in intact animals indicate that the NOP-selective antagonist JTC-801 exhibits a pronounced analgesic effect, both in mechanical and thermal nociception. Its administration alone significantly increased the pain threshold ($p<0.001$) compared to the control group throughout the observation period (214±2.45, 176±2.45, 154±2.45 g/cm² at 10, 20, and 30 min, respectively) (fig. 10).

Co-administration of JTC-801 with N/OFQ(1-13)NH_2 produced a statistically significant decrease in pain threshold compared to the peptide alone at 20 and 30 min ($p<0.001$), with values of 202±3.74, 156±2.45, 124±2.45 g/cm², compared to 306±8.12, 210±5.48, 122±3.74 g/cm² for N/OFQ(1-13)NH_2 alone (figs. 4 and 10). These results demonstrate that JTC-801 antagonizes the analgesic effect of nociceptin.

The analogue [Orn^9] alone significantly increased the pain threshold (306±2.45, 226±5.1, 120±4.47 g/cm²), whereas co-administration with JTC-801 significantly reduced the pain threshold at 10 and 20 min ($p<0.001$) and at 30 min ($p<0.01$). The measured values were markedly lower (188±3.74, 156.8±1.02, 138.8±0.8 g/cm²), indicating antagonism of the peptide's analgesic effect (figs. 4 and 10).

The analogue [Orn^9, Orn^{13}] exhibited the highest pain threshold values when administered alone (318±3.74, 218±3.74, 120±3.16 g/cm²) (fig. 4). In combination with JTC-801, it significantly decreased the pain threshold ($p<0.001$) throughout the entire observation period (189.6±1.33, 152±1.41, 115.6±1.33 g/cm²), confirming the antagonistic role of JTC-801 (fig. 10).

All peptides maintained a statistically significantly higher pain threshold compared to the control group ($p<0.001$), indicating partial preservation of analgesic effect despite the presence of the antagonist. It is known that NOP receptors can mediate both pronociceptive and antinociceptive effects depending on dose, receptor localization, and physiological context. This is well documented in the literature. Toll et al. reported that i.c.v. administration of nociceptin induces pronociception, while i.t. administration produces analgesia in chronic pain models. Similar findings by Hao et al. demonstrated that supraspinal activation of NOP receptors can suppress μ-opioid agonist effects, whereas spinal activation leads to a significant reduction in allodynia and hyperalgesia.

In the context of the present study, this functional plasticity of NOP receptors may explain the partial preservation of analgesia in the PP test during co-administration of JTC-801 with the peptides, as well as the hyperalgesia observed in the HP test. Results from the HP test confirmed observations from the PP model. JTC-801 alone significantly prolonged latency throughout the observation period compared to the control group ($p<0.001$; $p<0.01$), with values of 9.6±0.06, 6.7±0.10, and 5.8±0.06 sec, inducing analgesia in intact animals (fig. 11).

Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and the analogues significantly shortened latency ($p<0.01$; $p<0.001$) throughout the observation period (fig. 11). JTC-801 with N/OFQ(1-13)NH_2 reduced latency (5.38±0.06, 5.96±0.04, 4.76±0.07 sec) compared to peptide alone (8.4±0.25, 6.4±0.4, 4.7±0.14 sec), indicating antagonism of the peptide's analgesic effect (figs. 5 and 11). The analogue [Orn^9] alone prolonged latency (9.1±0.16, 5.88±0.15, 5.56±0.07 sec).

When co-administered with JTC-801, latency was significantly shortened (4.72±0.10, 4.46±0.09, 3.84±0.08 sec), indicating antagonism of analgesia and induction of hyperalgesia (figs. 5 and 11). The analogue [Orn^9, Orn^{13}] produced pronounced analgesic effects when administered alone (9.56±0.12, 6.22±0.09, 4.34±0.07 sec), but co-administration with JTC-801 significantly reduced latency (4.72±0.10, 4.32±0.08, 3.8±0.07 sec), confirming the antagonistic effect of JTC-

801 on peptide-induced analgesia (figs. 5 and 11). In the present study, partial analgesia was observed in the PP test, whereas hyperalgesia was noted in the HP test. The analogues [Orn^9] and [Orn^9, Orn^{13}] exhibited pronounced pronociceptive effects, shortening latency compared to the control group at 10 min ($p<0.01$). This may be explained by an increased affinity for the NOP receptor, as described by Preti, Calò, and Guerrini. These authors emphasize that modifications in the C-terminal region of N/OFQ, including ornithine substitutions, alter the pharmacodynamic profile of the peptide by stabilizing the α-helical structure and improving receptor selectivity. Data from the literature support the observed effects of JTC-801 in the present study. Tamai et al. and Suyama et al. demonstrated that JTC-801 is effective in inflammatory pain, suppressing allodynia and hyperalgesia. This corresponds to our observations of pronounced analgesic effects under both mechanical and thermal stimulation. Mabuchi et al. further reported that JTC-801 reduces pain by inhibiting nitric oxide production, suggesting a multimodal mechanism involving neuroimmune modulation. Our results are consistent with those of Yamada et al., who showed that JTC-801 possesses high selectivity for the NOP receptor, antagonizing nociceptin-induced inhibition of cAMP signaling. JTC-801 induces analgesia by prolonging latency in the HP test and reducing the number of nociceptive responses in the second phase of the formalin test in rodents. The analgesic effect is most pronounced during the initial minutes after administration and gradually diminishes thereafter. PP test results indicate that 1hIS significantly increased the pain threshold compared to the control group throughout the observation period ($p<0.001$), thereby inducing analgesia (fig. 12).

Stress-induced analgesia (SIA) is mediated by activation of descending inhibitory pathways involving endogenous opioids, GABA, glutamate, monoamines, endocannabinoids, and the NOP receptor system. Pola et al. and Toll et al. highlighted that the NOP receptor system plays a significant role in pain modulation and stress responses, with NOP antagonists being considered potential therapeutic agents for mitigating the adverse effects of stress on pain perception. Co-administration of JTC-801 with nociceptin and its analogues following stress resulted in a statistically significant decrease in pain threshold compared to the 1hIS group ($p<0.001$), indicating that JTC-801 blocks stress-induced analgesia (fig. 12). This finding aligns with the data of Delaney et al., who demonstrated that NOP receptor blockade with JTC-801 alters the neuroendocrine response to acute stress, including HPA axis activity and NOP receptor gene expression. The pain threshold remained higher than that of the control group, suggesting partial preservation of analgesic effects, likely due to the activity of other neuromodulatory systems involved in SIA. As highlighted by Butler & Finn, SIA is a multimodal process involving endogenous opioids, endocannabinoids, GABA,

glutamate, oxytocin, and other neurotransmitters, activated via descending inhibitory pathways from the cerebral cortex to the spinal cord. Pola et al. emphasize that the NOP receptor system is only one component of the complex neuronal network regulating stress – pain interactions, and that NOP receptor blockade does not completely abolish SIA, consistent with the partial preservation of analgesia observed in the present study. Hot Plate test results showed that 1hIS significantly increased latency only at 10 min ($p<0.05$), consistent with the transient activation of endogenous analgesic mechanisms characteristic of SIA (fig. 13). In the HP test, administration of JTC-801 with the analogues led to a statistically significant shortening of latency for [Orn^9] ($p<0.001$) and [Orn^9, Orn^{13}] ($p<0.05$), confirming the antagonistic role of JTC-801 and suggesting pronociceptive activity of the peptides under stress conditions (fig. 13).

Activation of NOP receptors leads to inhibition of Cav2.2 channels via G-protein–dependent mechanisms, reducing neuronal excitability and the release of pain mediators. Blockade of these receptors with JTC-801 may interrupt this inhibitory effect, resulting in restored pain sensitivity, especially under stress conditions when the NOP system is activated. NOP receptors are known to participate in the regulation of the HPA axis, which is activated during stress. Activation of central NOP receptors modulates corticosteroid release, which contributes to endogenous analgesia characteristic of acute stress. According to Castro et al., the stress system, including CRH and noradrenergic pathways, participates in HPA axis regulation, and glucocorticoids released in response to stress exert negative feedback on CRH and ACTH, thereby modulating pain sensitivity. Blockade of NOP receptors with JTC-801 may disrupt this regulation, reducing corticosteroid release and suppressing activation of descending inhibitory pathways, leading to decreased SIA.

This is consistent with our PP and HP test results, where co-administration of JTC-801 with nociceptin and its analogues following stress resulted in a statistically significant decrease in pain threshold compared to the 1hIS group, indicating that NOP receptor blockade compromises endogenous analgesia.

It is known that acute stress activates descending inhibitory pathways, including endogenous opioids, which suppress pain sensitivity. NOP receptors interact with the opioid system, modulating the release of neurotransmitters and hormones involved in pain regulation. Blockade of NOP receptors with JTC-801 may induce an imbalance between opioid and nociceptinergic activity, as reflected in our results by partial preservation of analgesia but also potential pronociceptive effects of the analogues, particularly during the early minutes following stress. This aligns with findings by al'Absi, Nakajima, and Bruehl, who demonstrated that blockade of the opioid system with antagonists (e.g., naltrexone) abolishes SIA, especially in response to thermal and mechanical pain stimuli. The authors

emphasize that the endogenous opioid system is a key mediator of SIA, but its interaction with other systems, including NOP, can modulate pain responses depending on stress type and pain modality.

The observed effects in this study are consistent with data from other stress and pain models. In chronic stress models, such as repeated immobilization or social isolation, stress-induced hyperalgesia (SIH) is often observed, in contrast to 1hIS, which induces SIA. According to Pola et al., the NOP receptor system participates in both SIA and SIH, with the effect dependent on stress duration and intensity. In a post-traumatic stress disorder (PTSD) model, persistent mechanical allodynia and thermal hyperalgesia are observed up to 28 days post-stress. Zhang et al. reported increased nociceptin levels in the CNS and serum, along with reduced glucocorticoid secretion, indicating HPA axis dysregulation. In our cold stress model, the analgesic effects of the [Orn^9] and [Orn^9, Orn^{13}] analogues are mediated by opioid, nociceptinergic, and nitric oxide systems. Delaney et al. demonstrated that acute and repeated stress alter NOP receptor expression in limbic structures such as the hypothalamus and hippocampus, suggesting that the NOP system is highly sensitive to stress and that pharmacological blockade with JTC-801 can disrupt central mechanisms involved in pain modulation.

The results of the present study indicate that N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] suppress immobilization-induced SIA following NOP receptor blockade, displaying variable anti-stress activity depending on the type of pain test. In the PP test, 1hIS significantly increased the pain threshold ($p<0.001$) compared to the control group (up to 95% at 10 min), indicating the presence of SIA. Co-administration of JTC-801 with N/OFQ(1-13)NH_2 and its analogues resulted in a statistically significant decrease in the pain threshold throughout the observation period ($p<0.01$; $p<0.001$) compared to 1hIS. Specifically, N/OFQ(1-13)NH_2 reduced the pain threshold by 32% at 10 min; [Orn^9] by 26.45% and [Orn^9, Orn^{13}] most strongly suppressed SIA, by 35% at 10 min compared to 1hIS (fig. 12).

In the HP test, the SIA effect was weaker and more transient, with a statistically significant increase in latency time compared to control ($p<0.05$) observed only at 10 min (~21%). Co-administration of JTC-801 with nociceptin and its analogues after stress significantly shortened latency time at 10 min compared to 1hIS ($p<0.05$ for [Orn^9, Orn^{13}] and $p<0.001$ for [Orn^9]). [Orn^9] suppressed SIA by ~25.7%, whereas [Orn^9, Orn^{13}] and N/OFQ(1-13)NH_2 exhibited weaker effects (~15%). At 20 and 30 min, the effect was minimal or absent (fig. 13).

Our results indicate that the NOP receptor system participates in the modulation of SIA, and the anti-stress activity of N/OFQ and its analogues depends on the type of nociceptive stimulus.

Results from the PP test indicate that chronic immobilization stress (ChIS) significantly increased the pain threshold at 10 min ($p<0.001$) and 20 min ($p<0.05$) compared to the control group, inducing analgesia (fig. 14). Similarly, the HP test showed prolonged latency at 10 min ($p<0.001$) and 20 min ($p<0.01$) relative to controls (fig. 15). This corresponds to stress-induced analgesia (SIA), in which acute or early-phase chronic stress activates endogenous antinociceptive mechanisms. Under conditions of prolonged stress, a transition to stress-induced hyperalgesia (SIH) may occur, particularly with extended exposure or pharmacological intervention, as reported by Polá et al.

Our results demonstrate that administration of JTC-801 immediately after stress led to a significant decrease in both pain threshold and latency compared to ChIS and control groups throughout the observation period ($p<0.001$), inducing hyperalgesia and confirming the hypothesis that NOP receptor blockade disrupts endogenous antinociceptive regulation (figs. 14 and 15).

It is well established that chronic stress activates the HPA axis, elevates glucocorticoid levels, and induces central sensitization via inflammatory and neuroendocrine mediators such as CRF, IL-1β, and TNF-α. Neuroinflammatory processes in the spinal cord, described by Rivat and colleagues, trigger sensory hypersensitivity and long-lasting hyperalgesia, which may also account for our observations. Scheich et al. demonstrated that chronic stress induces mechanical hyperalgesia via capsaicin-sensitive neurons, further supporting the hypothesis of central sensitization and the involvement of peripheral nociceptive mechanisms. Data from Feng et al. indicate that chronic immobilization leads to tolerance to the anti-nociceptive effects of stress through reduced expression of POMC and β-endorphin, along with activation of inflammatory signaling pathways such as MAPK, COX-2, and iNOS. These alterations in both central and peripheral nervous systems contribute to desensitization of antinociceptive systems and increased pain sensitivity. In the present study, these mechanisms may explain the diminished efficacy of nociceptin and its analogues under chronic stress, as well as the enhanced hyperalgesia observed following NOP receptor blockade.

Combined administration of JTC-801 with N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] following stress resulted in a statistically significant reduction in pain threshold (PP test) compared to ChIS ($p<0.001$) at 10 min, indicating that NOP receptor blockade attenuates the analgesic effect of the peptides. At 20 min, a significant decrease in pain threshold ($p<0.01$) was observed for N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}], and at 30 min, only for [Orn^9, Orn^{13}] ($p<0.05$) (fig. 14). In the HP test, N/OFQ(1-13)NH_2 and the analogues showed a statistically significant shortening of latency compared to both ChIS and control groups throughout the entire observation period ($p<0.001$), reflecting increased pain sensitivity and hyperalgesia. These results indicate that NOP receptor

blockade completely suppresses the analgesic effect of the peptides under chronic stress conditions (fig. 15).

NOP receptors and N/OFQ are known to be expressed in the hypothalamus and limbic system – key structures for stress response regulation. Acute restraint stress leads to reduced expression of ppN/OFQ and NOP mRNA in the hippocampus and hypothalamus, whereas repeated stress induces compensatory changes, including increased expression in the BNST and reticular thalamus. This suggests an adaptive role for the NOP system, which can be disrupted by pharmacological blockade. NOP receptors play a crucial role in pain regulation by inhibiting neurotransmitter release and modulating neuronal excitability in the spinal cord. Their blockade with JTC-801 disrupts the balance between antinociceptive and pronociceptive signals, manifesting as hyperalgesia.

The application of JTC-801 in our study highlights the role of NOP receptors in stress-related pain modulation. In post-traumatic stress models, Zhang et al. demonstrated that JTC-801 exhibits both analgesic and anxiolytic effects. In rats exposed to a single prolonged stress, JTC-801 significantly reduced mechanical allodynia and thermal hyperalgesia, normalized corticosterone levels, and blocked the stress-induced increase in NOP receptor expression in the amygdala and PAG. These findings emphasize the modulatory role of the NOP system under stress and suggest that JTC-801 could be used in conditions combining pain and anxiety disorders.

In the present study, JTC-801 induced hyperalgesia in animals subjected to ChIS. This discrepancy indicates that NOP receptor modulation may vary depending on the type and duration of stress and the physiological state of the animals. Differences between models (PTSD vs. ChIS) suggest that the NOP system participates in distinct mechanisms of pain regulation, warranting further investigation to clarify its role. Studies by Porro & Carli and Molina et al. have shown that different forms of physical restraint – immobilization and restraint stress lead to different pain responses, including hypoalgesia and hyperalgesia, depending on the duration and intensity of exposure. This supports the choice of model in the present study and provides a rationale for the observed results.

Results from the two nociceptive tests indicate that in the HP test, the hyperalgesic effect of combined administration of the NOP-selective antagonist with N/OFQ(1-13)NH_2 and its analogues is stronger and more sustained. For all peptides, latency was shortened compared to the control group, whereas the mechanical pain threshold in the PP test was significantly reduced only at the 10-minute time point. In the combined administration of JTC-801 with [Orn^9, Orn^{13}], latency was reduced by 31.4% compared to control, whereas the pain threshold decreased by only 4.8%. A similar trend was observed for the other peptide variants, with the thermal test showing, on average, a 25–30% stronger

hyperalgesic effect. This effect persisted over time, suggesting a higher sensitivity of thermal nociception to NOP receptor blockade under chronic stress conditions (figs. 14 and 15).

3. Effect of the non-selective opioid receptor antagonist naloxone (Nal) on the analgesic effects of nociceptin N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] in:

3.1. animals subjected to 1hIS

Paw Pressure test

The 1hIS group showed a statistically significant increase in pain threshold compared to the control group ($p<0.001$) throughout the entire observation period, confirming the presence of stress-induced analgesia (SIA).
Combined administration of Nal with nociceptin and its analogues – [Orn^9] and [Orn^9, Orn^{13}] after stress resulted in a statistically significant reduction in pain threshold compared to the 1hIS group ($p<0.001$) for the entire observation period. Compared to the control group, pain thresholds remained significantly elevated ($p<0.01$; $p<0.001$) throughout the study period (fig. 16).

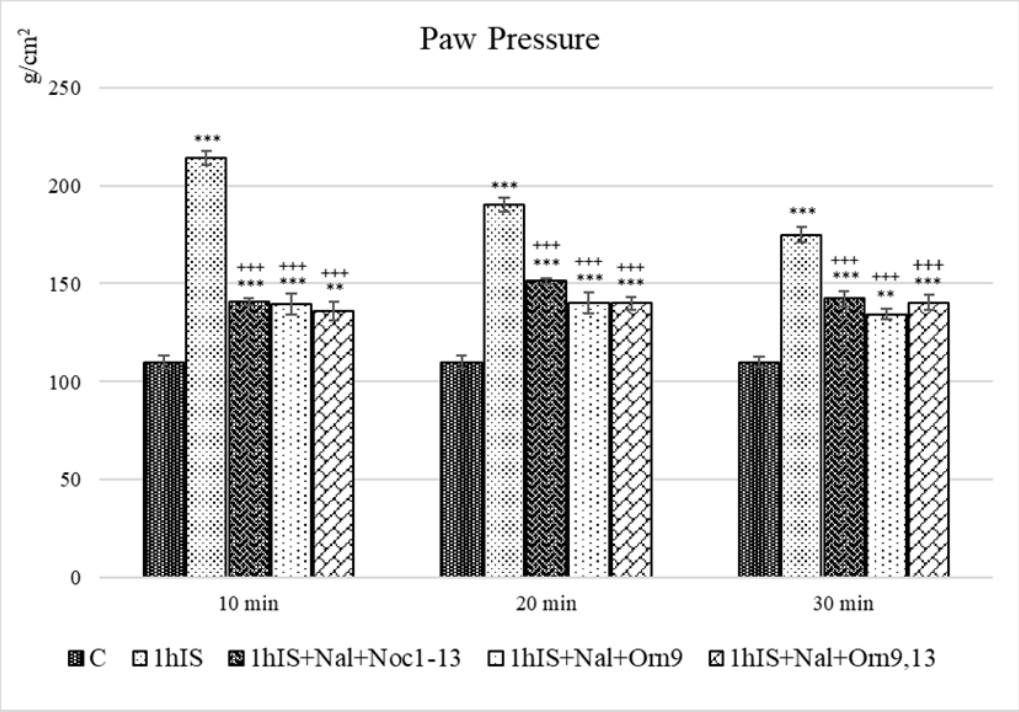

*Figure 16. Effects of Nal (1 mg/kg, i.p.) administered with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on pain threshold after 1hIS. Data are presented as mean ± S.E.M.; *$p<0.01$; $p<0.001$ vs. control; $^{+++}p<0.001$ vs. 1hIS.*

Hot Plate test

The 1hIS group showed a statistically significant increase in latency compared to the control group ($p<0.01$) at the 10th minute of the test.

Combined administration of Nal with nociceptin and its analogues resulted in a statistically significant decrease in latency compared to the 1hIS group at the 10th minute ($p<0.01$) for N/OFQ(1-13)NH_2, ($p<0.05$) for [Orn^9], and ($p<0.001$) for [Orn^9, Orn^{13}]. The analogue [Orn^9, Orn^{13}] also significantly decreased latency compared to the control group at the 10th minute ($p<0.05$). No statistically significant differences between groups were observed at the 20th and 30th minutes of the test (fig. 17).

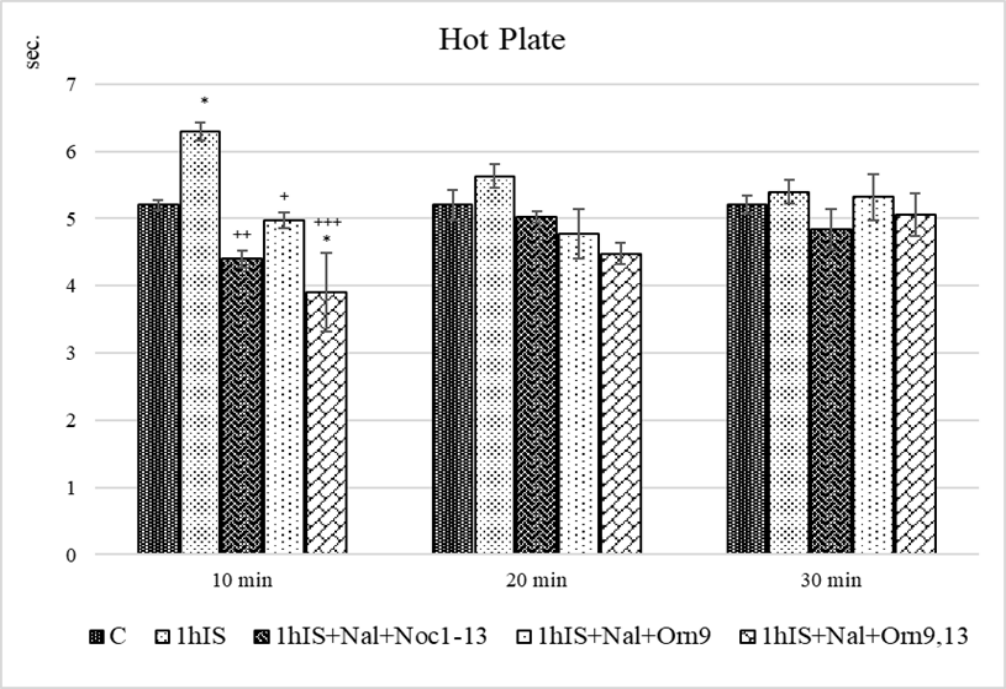

Figure 17. Effects of Nal (1 mg/kg, i.p.) administered with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on HP latency after 1hIS. Data are presented as mean ± S.E.M.; $p<0.05$ vs. control; $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ vs. 1hIS.

3.2. Effects of the non-selective opioid receptor antagonist Nal on the analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] in animals subjected to ChIS

Paw Pressure test

Chronic immobilization stress (ChIS) significantly increased the pain threshold at the 10th minute ($p<0.001$) and the 20th minute ($p<0.01$) compared to the control group.

Administration of Nal after stress led to a statistically significant decrease in pain threshold compared to ChIS alone ($p<0.001$) at the 10th and 20th minutes. Combined administration of Nal with nociceptin N/OFQ(1-13)NH_2 and its analogues after stress resulted in a statistically significant reduction of pain threshold compared to ChIS at the 10th minute ($p<0.001$).

At the 20th minute, a significant decrease in pain threshold was observed for N/OFQ(1-13)NH_2 ($p<0.05$) and [Orn^9, Orn^{13}] ($p<0.01$) compared to the ChIS group. No statistically significant differences between groups were observed at the 30th minute (fig. 18).

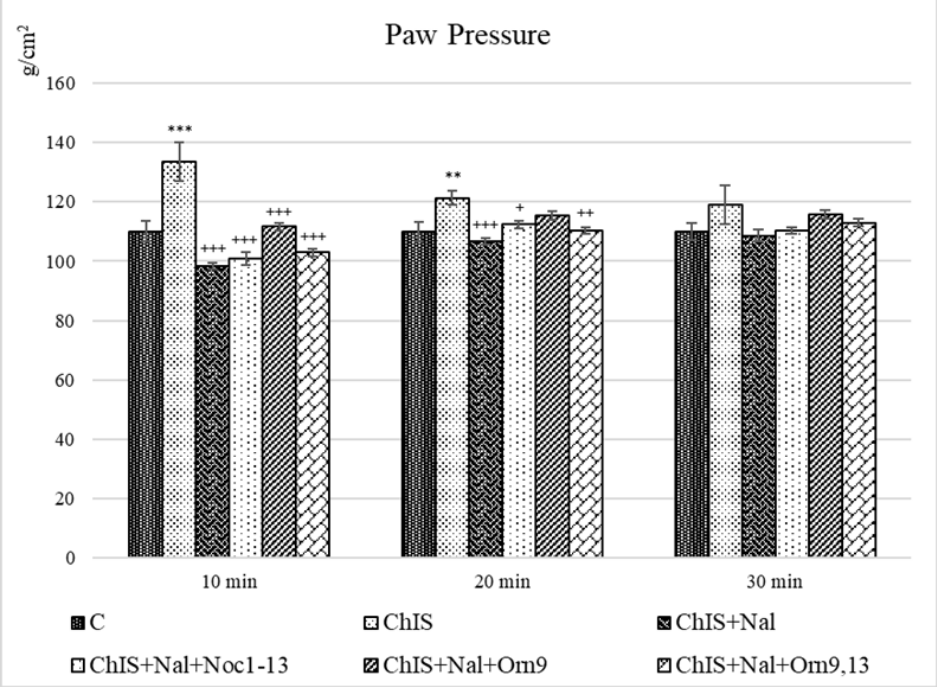

*Figure 18. Effects of Nal (1 mg/kg, i.p.) administered with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 µg/kg, i.p.) on pain threshold after ChIS. Data are presented as mean ± S.E.M.; **p<0.01; *p<0.001 vs. control; $^{++}$p<0.01; $^{+++}$p<0.001 vs. ChIS.*

Hot Plate test

Chronic immobilization stress (ChIS) significantly increased latency compared to the control group at the 10th minute ($p<0.001$) and 20th minute ($p<0.01$).

Administration of Nal immediately after stress led to a statistically significant reduction in latency compared to both the control and ChIS groups for the entire observation period ($p<0.001$), indicating pronounced hyperalgesia.

Combined administration of Nal with N/OFQ(1-13)NH_2 and its analogues [Orn^9] and [Orn^9, Orn^{13}] after stress also resulted in a statistically significant decrease in latency compared to control and ChIS ($p<0.001$) (fig. 19).

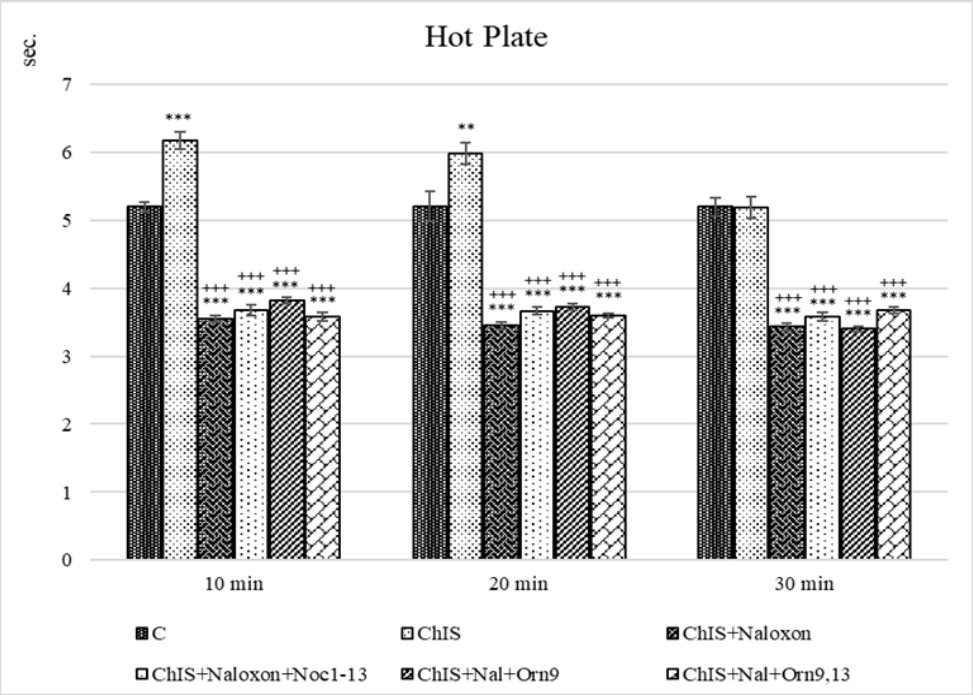

*Figure 19. Effects of Nal (1 mg/kg, i.p.) administered with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] (10 μg/kg, i.p.) on HP latency after ChIS. Data are presented as mean ± S.E.M.; **p<0.01; *p<0.001 vs. control; $^{+++}$p<0.001 vs. ChIS.*

3.3. Discussion

The present study aimed to evaluate the influence of the non-selective opioid receptor antagonist naloxone (Nal) on the analgesic activity of nociceptin N/OFQ(1-13)NH_2 and its structural analogues [Orn^9] and [Orn^9, Orn^{13}] in animals subjected to acute and chronic immobilization stress.

Naloxone is a non-selective antagonist of classical opioid receptors – μ, δ, and κ widely used both clinically and in experimental models to assess the functional activity of the endogenous opioid system.

It is well established that immobilization stress induces increased antinociception in tail-flick, hot-plate, and formalin tests. Studies by Amir and Amit have shown

that naloxone reduces stress-induced inhibition of responses in tail-flick and hot-plate tests, confirming the involvement of the opioid system.

Our results show that N/OFQ(1-13)NH_2 and its analogues, administered together with naloxone after 1hIS, significantly decreased the pain threshold ($p<0.001$) and shortened latency ($p<0.01$ for N/OFQ(1-13)NH_2; $p<0.05$ for [Orn^9]; $p<0.001$ for [Orn^9, Orn^{13}]) compared to the stress-exposed group, indicating that naloxone antagonizes SIA and the analgesic effects of the peptides (figs. 16 and 17).

A characteristic feature of the nociceptin system is its ability to antagonize classical opioid effects. Peptides of this class can inhibit morphine analgesia, reduce tolerance to levorphanol, and may induce withdrawal symptoms in morphine-dependent animals. This suggests that nociceptin and its analogues interact with the opioid system in a complex, context-dependent manner, here suppressing immobilization-induced SIA.

Comparative analysis of the two nociceptive tests (PP and HP) shows that the effect of co-administration of naloxone with nociceptin and its analogues after 1hIS is more pronounced in the thermal test, particularly during the early minutes, where latency is significantly reduced below control values. The most pronounced hyperalgesia is observed with Nal+[Orn^9, Orn^{13}] at 10 minutes (~25%), followed by Nal+N/OFQ(1-13)NH_2 (~15%) and Nal+[Orn^9] (~4%). In the PP test, pain threshold values in all groups are significantly higher ($p<0.001$) than controls (~29%), without evidence of hyperalgesia. These results confirm the involvement of the opioid system in the effects of the peptides under 1hIS conditions.

Chronic immobilization stress (ChIS) is a well-established experimental model widely used to induce physiological and behavioral changes, including depression-like behavior, anxiety, and alterations in pain sensitivity. Multiple studies have shown that ChIS leads to significant neurochemical and structural changes in brain regions such as the hippocampus and prefrontal cortex, including dysregulation of dopaminergic and GABAergic systems, as well as activation of the HPA axis and increased neuroinflammatory activity.

Chronic stress is associated with changes in the expression and sensitivity of μ-, δ-, and κ-opioid receptors. Typically, μ- and δ-receptor activity is reduced, while κ-receptor activity is compensatorily increased. This dysregulation of the endogenous opioid system results in a diminished analgesic response and increased pain sensitivity, particularly during prolonged stress exposure.

Administration of naloxone immediately after chronic stress (ChIS) resulted in a significant reduction of the pain threshold at 10 and 20 minutes ($p<0.001$) compared to the ChIS group (fig. 18), as well as a shortening of latency compared to both ChIS and control groups ($p<0.001$) throughout the observation period (fig. 19). These results indicate that opioid receptor blockade completely suppresses

stress-induced analgesia and induces hyperalgesia, confirming the key role of the endogenous opioid system in modulating pain sensitivity under chronic stress conditions. Co-administration of naloxone with N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] significantly decreased the pain threshold ($p<0.001$) compared to the ChIS group and shortened latency ($p<0.001$) compared to ChIS and control groups in the HP test, inducing hyperalgesia under opioid blockade conditions (figs. 18 and 19). According to literature data, nociceptin can inhibit opioid-mediated analgesia and induce hyperalgesia under stress conditions. This action is mediated through activation of NOP receptors, which are structurally similar to classical opioid receptors but possess distinct pharmacological properties.

Naloxone, as a non-selective opioid receptor antagonist, blocks the action of endogenous opioids and unmasks the role of other neuromodulatory systems, including the nociceptin system. The interaction between the opioid and NOP receptor systems is critical for understanding the mechanisms of pain perception under chronic stress. In the PP test, the stress-exposed group increased the pain threshold by 21.5% compared to controls, confirming the presence of SIA. Administration of naloxone reduced the pain threshold by 26.5% compared to the ChIS group at 10 minutes, indicating blockade of opioid-mediated analgesia. Co-administration of naloxone with N/OFQ(1-13)NH_2 and its analogues also produced a significant decrease in the pain threshold at 10 and 20 minutes compared to ChIS. In the HP test, naloxone shortened latency by ~42% compared to the ChIS group. Co-administration of nociceptin and its analogues with naloxone reduced latency by 38–41% compared to the stress group. These results indicate that thermal nociception is more sensitive to opioid blockade and that the peptides significantly reduce latency compared to controls, inducing hyperalgesia.

4. Influence of the nitric oxide system on the analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}], via L-NAME (NOS inhibitor) and L-arginine (NO precursor) in:

4.1. Animals subjected to 1hIS

Paw Pressure test

The 1hIS-exposed group exhibited a statistically significant increase in pain threshold compared to controls ($p<0.001$) throughout the observation period. Administration of L-arginine after stress resulted in a statistically significant increase in pain threshold ($p<0.001$) compared to controls and ($p<0.05$; $p<0.001$) compared to the 1hIS group at 10 and 20 minutes. Co-administration of L-arginine

with nociceptin and its analogues produced a statistically significant decrease in pain threshold ($p<0.001$) compared to both the 1hIS group and controls throughout the study period (fig. 20).

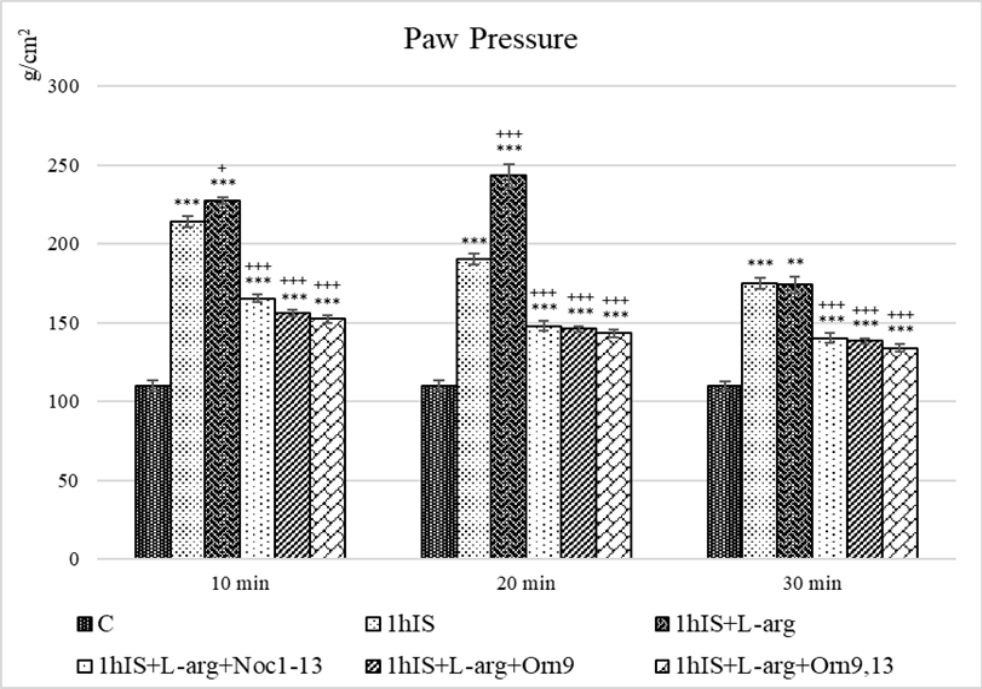

*Figure 20. Effects of L-arginine (1 mg/kg, i.p.) co-administered with N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on pain threshold after 1hIS. Data are presented as mean ± S.E.M.; **p<0.01; ***p<0.001 vs. control; $^{+}$p<0.05; $^{++}$p<0.01; $^{+++}$p<0.001 vs. 1hIS.*

Hot Plate test

Acute immobilization stress (1hIS) significantly increased the latency time ($p<0.05$) compared to the control group at the 10th minute. Administration of L-arginine (L-arg) after stress significantly prolonged latency time ($p<0.001$) compared to both control and 1hIS groups throughout the entire observation period. In the groups 1hIS+L-arg+N/OFQ(1-13)NH_2, 1hIS+L-arg+[Orn^9], and 1hIS+L-arg+[Orn^9, Orn^{13}], a statistically significant reduction in latency time was observed ($p<0.01$; $p<0.001$) compared to 1hIS throughout the test period, and ($p<0.05$; $p<0.01$; $p<0.001$) compared to the control group at the 20th and 30th minutes of the study (fig. 21).

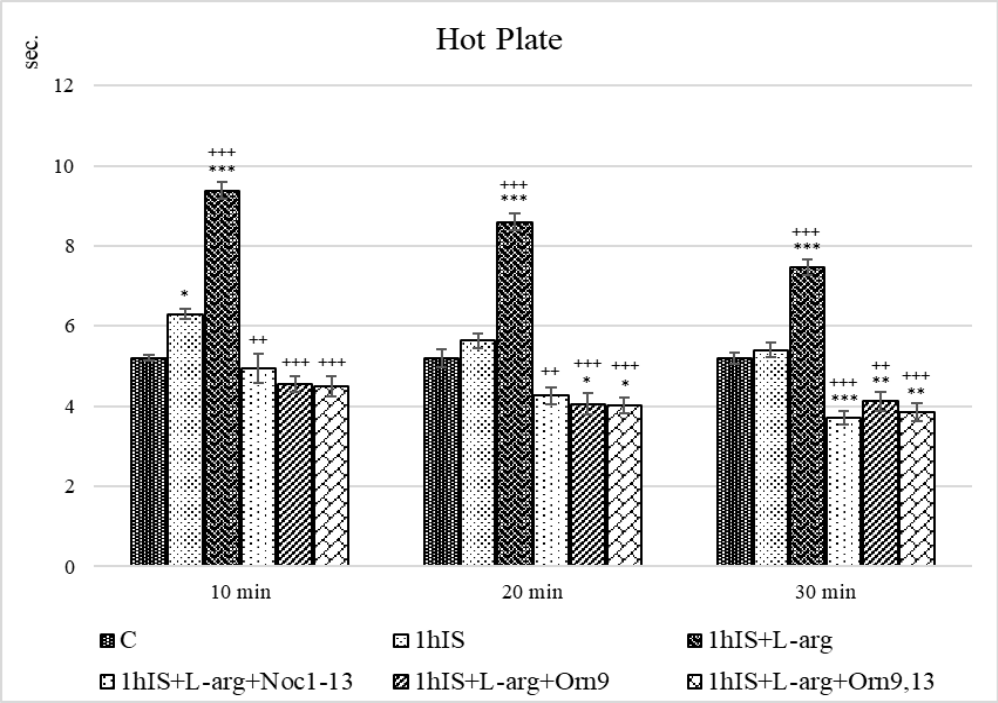

*Figure 21. Effects of L-arginine (L-arg, 1 mg/kg, i.p.) administered together with N/OFQ(1-13)NH₂ and analogues – [Orn⁹] and [Orn⁹, Orn¹³] on HP latency after 1hIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 versus control; ⁺⁺p<0.01; ⁺⁺⁺p<0.001 versus 1hIS.*

Paw Pressure test

Administration of L-NAME (10 mg/kg, i.p.) immediately after stress showed a statistically significant decrease in the pain threshold ($p<0.001$) compared to the 1hIS group throughout the entire observation period. Compared to the control group, a statistically significant increase in the pain threshold was observed at 10 min ($p<0.001$) and 20 min ($p<0.01$).

Combined administration of L-NAME with N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] significantly decreased the pain threshold ($p<0.001$) compared to 1hIS throughout the observation period. Compared to the control group, a statistically significant increase in the pain threshold was observed at 10 and 20 min ($p<0.001$). At 30 min, a statistically significant increase in the pain threshold compared to control was observed for L-NAME + N/OFQ(1-13)NH_2 ($p<0.001$), L-NAME + [Orn^9] ($p<0.01$), and L-NAME + [Orn^9, Orn^{13}] ($p<0.05$) (fig. 22).

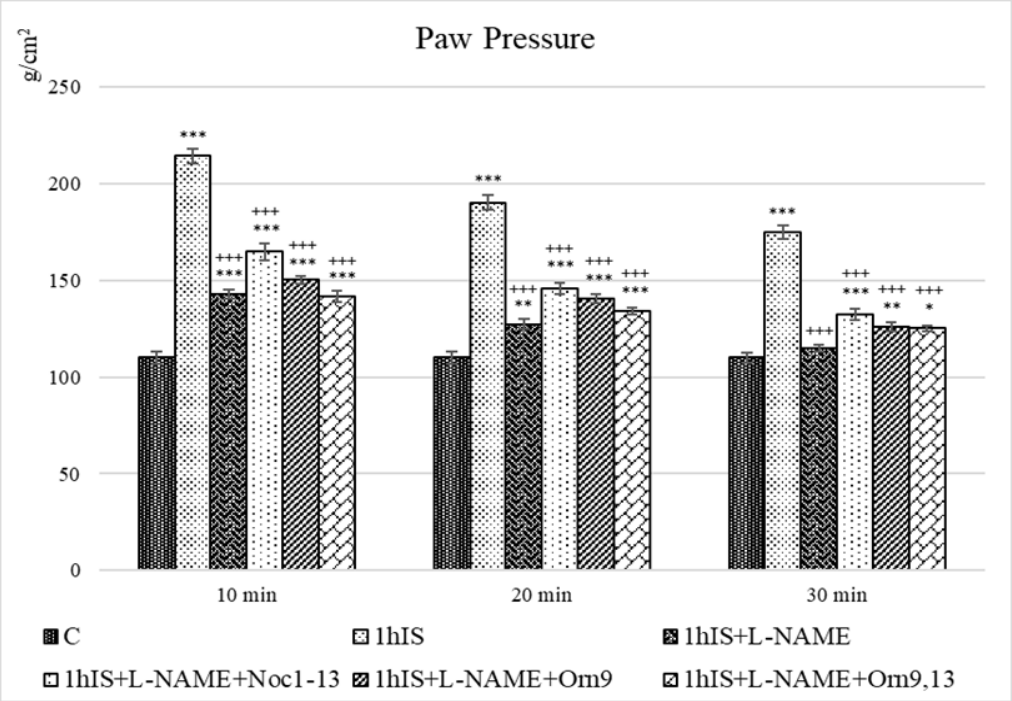

*Figure 22. Effects of L-NAME (10 mg/kg, i.p.) when co-administered with N/OFQ(1–13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] on pain threshold following 1hIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 versus control; $^{+++}$p<0.001 versus 1hIS.*

Hot Plate test

Administration of L-NAME after stress resulted in a statistically significant shortening of latency compared to the control group ($p<0.01$) at the 10th minute and ($p<0.001$) at the 20th and 30th minutes, and compared to the 1hIS group ($p<0.001$) for the entire testing period.

Co-administration of L-NAME with N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] led to a statistically significant shortening of latency ($p<0.001$) compared to both the control and 1hIS groups throughout the testing period (fig. 23).

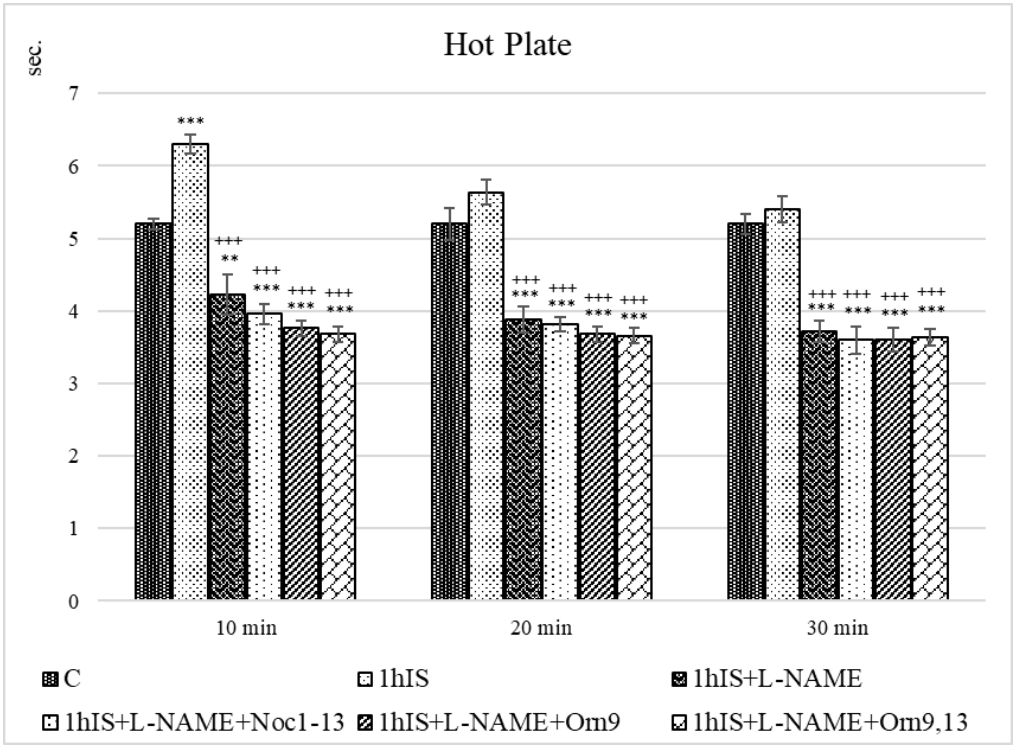

*Figure 23. Effects of L-NAME (10 mg/kg, i.p.) co-administered with N/OFQ(1–13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] on HP latency after 1hIS. Data are presented as mean ± S.E.M.; **p<0.01; ***p<0.001 vs. control; $^{+++}$p<0.001 vs. 1hIS.*

4.2. Influence of the nitric oxide system on the analgesic effects of N/OFQ(1-13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] via L-NAME and L-arginine in animals subjected to ChIS

Paw Pressure test

In the ChIS group, a statistically significant increase in the pain threshold was observed at the 10th minute of the test ($p<0.01$) compared to the control group.

Administration of L-arginine (L-arg) alone immediately after stress led to a statistically significant increase in the pain threshold compared to control ($p<0.001$) and compared to the ChIS group ($p<0.01$; $p<0.001$) for the entire period of the study.

At the 10th minute, co-administration of L-arg with the peptides led to a decrease in the pain threshold, but no statistically significant differences were observed compared to the ChIS group. At the 20th minute, in the L-arg + [Orn^9, Orn^{13}] group, a statistically significant increase in the pain threshold was observed compared to control ($p<0.01$). Compared to ChIS, the pain threshold was increased

but without statistical significance. At the 30th minute, the L-arg + [Orn^9] group showed a statistically significant increase in the pain threshold compared to control ($p<0.01$). Compared to ChIS, the pain threshold was increased but not statistically significant.

In the L-arg + N/OFQ(1-13)NH_2 group, a decrease in the pain threshold was observed compared to control and ChIS at the 20th and 30th minutes, but without statistical significance (fig. 24).

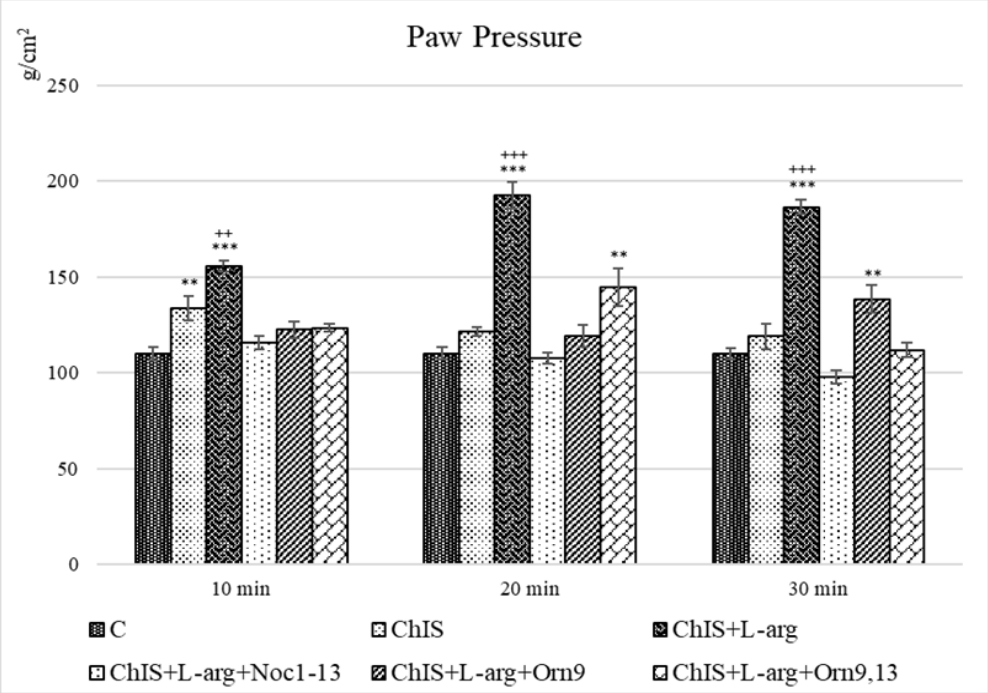

*Figure 24. Effects of L-arginine (1 mg/kg, i.p.) co-administered with N/OFQ(1–13)NH_2, [Orn^9], and [Orn^9, Orn^{13}] on the pain threshold after ChIS. Data are presented as mean ± S.E.M.; **p<0.01; ***p<0.001 vs. control; $^{++}$p<0.01; $^{+++}$p<0.001 vs. ChIS.*

Hot Plate test

The ChIS group showed a statistically significant prolongation of latency at 10 and 20 minutes ($p<0.01$ and $p<0.05$, respectively) compared to the control group, while at 30 minutes the latency was comparable to control.

Administration of L-arginine immediately after stress led to a statistically significant prolongation of latency compared to control ($p<0.001$) for the entire study period and compared to ChIS at 20 and 30 minutes ($p<0.001$). In the groups with combined administration – ChIS + L-arg + N/OFQ(1-13)NH_2, ChIS + L-arg + [Orn^9], and ChIS + L-arg + [Orn^9, Orn^{13}] a statistically significant shortening of latency was observed compared to control ($p<0.05$; $p<0.01$; $p<0.001$) and

compared to ChIS ($p<0.001$) at 10 and 20 minutes. At 30 minutes, the pain threshold remained reduced compared to control and ChIS, but without statistical significance (fig. 25).

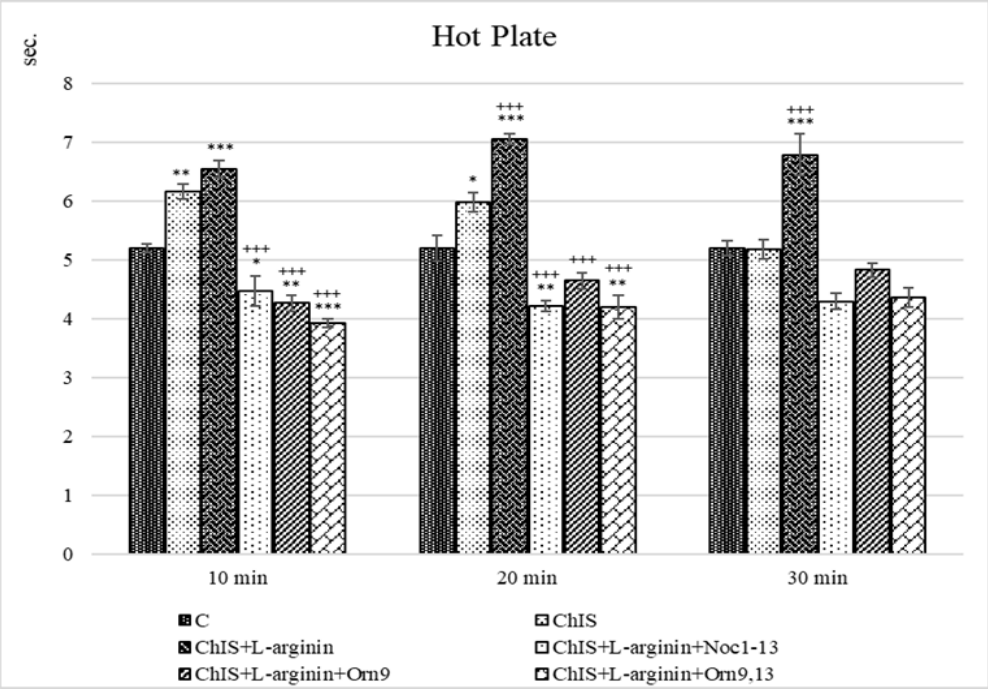

*Figure 25. Effects of L-arginine (1 mg/kg, i.p.) when co-administered with N/OFQ(1–13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] on HP latency after ChIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 vs. control; $^{+++}$p<0.001 vs. ChIS.*

Paw Pressure test

Administration of L-NAME after stress led to a statistically significant decrease in the pain threshold compared to ChIS ($p<0.001$) for the entire observation period, and compared to the control group ($p<0.001$) at 20 min and ($p<0.05$) at 30 min.

In the ChIS+L-NAME+N/OFQ(1-13)NH_2 group, a statistically significant decrease in the pain threshold was observed compared to both the control and ChIS groups ($p<0.001$) for the entire observation period. In the ChIS+L-NAME+[Orn^9, Orn^{13}] group, a statistically significant decrease in the pain threshold was observed compared to both the control and ChIS groups at 10 min and 20 min ($p<0.001$).

The ChIS+L-NAME+[Orn^9] group showed a statistically significant decrease in the pain threshold compared to ChIS ($p<0.01$) at 10 min. At 20 min, the pain threshold was significantly increased compared to the control ($p<0.001$)

and ChIS ($p<0.01$). At 30 min, the pain threshold remained elevated, but without statistically significant differences (fig. 26).

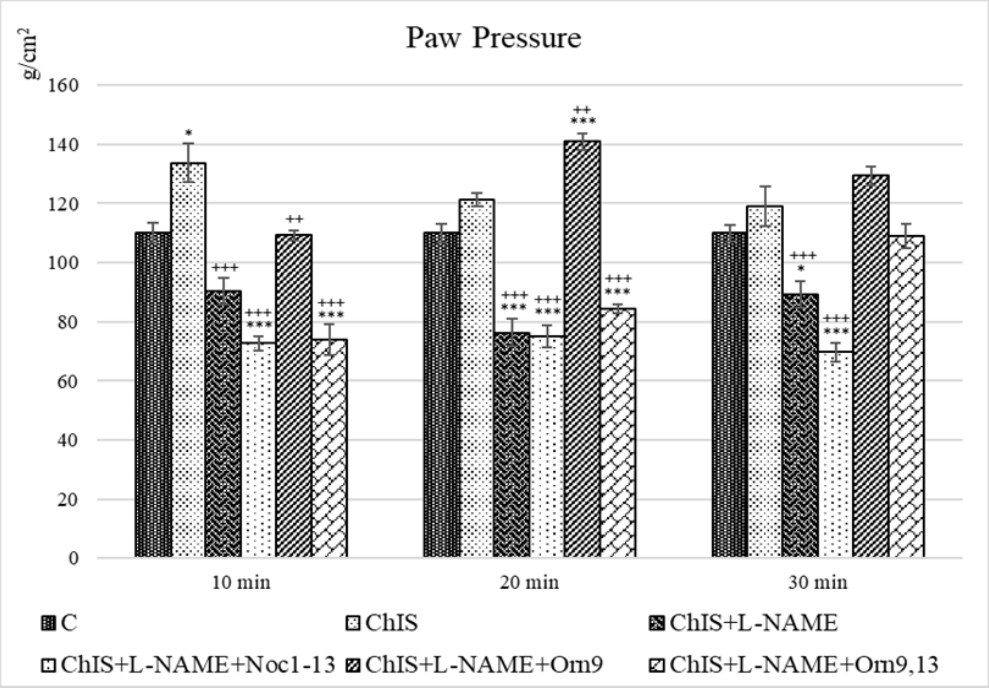

*Figure 26. Effects of L-NAME (10 mg/kg, i.p.) when co-administered with N/OFQ(1–13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on the pain threshold after ChIS. Data are presented as mean ± S.E.M.; *p<0.05; ***p<0.001 versus control; $^{++}$p<0.001; $^{+++}$p<0.001 versus ChIS.*

Hot Plate test

Administration of L-NAME after stress led to a statistically significant shortening of latency compared to the control group ($p<0.001$ at 10 min; $p<0.01$ at 20 and 30 min) and compared to the ChIS group ($p<0.001$) throughout the entire testing period.

Co-administration of L-NAME with N/OFQ(1-13)NH_2 resulted in a statistically significant reduction in latency compared to the control group ($p<0.001$ at 10 min; $p<0.01$ at 20 and 30 min) and compared to ChIS ($p<0.001$ at 10 and 20 min; $p<0.01$ at 30 min).

In the ChIS+L-NAME+[Orn^9] group, latency was significantly shortened compared to ChIS ($p<0.001$ at 10 min; $p<0.01$ at 20 min; $p<0.05$ at 30 min). Compared to the control group, latency was significantly reduced ($p<0.05$) at 30 min. In the ChIS+L-NAME+[Orn^9, Orn^{13}] group, latency was significantly

shortened compared to ChIS ($p<0.001$ at 10 and 20 min; $p<0.05$ at 30 min) and compared to the control group ($p<0.001$ at 10 min; $p<0.05$ at 30 min) (fig. 27).

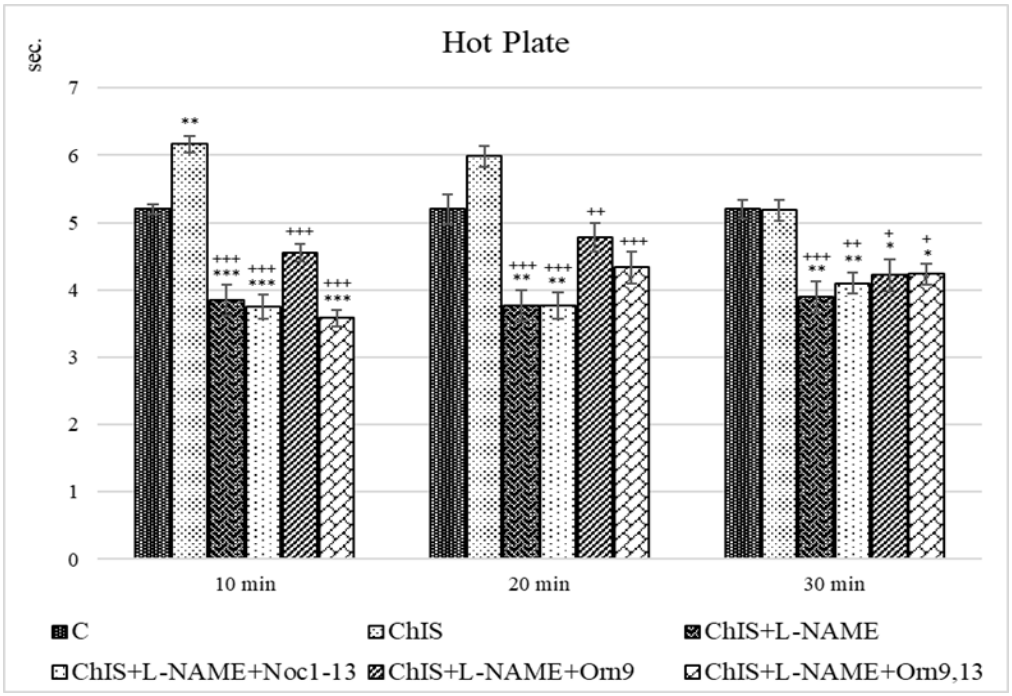

*Figure 27. Effects of L-NAME (10 mg/kg, i.p.) co-administered with N/OFQ(1–13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on HP latency following ChIS. Data are presented as mean ± S.E.M.; *p<0.05; **p<0.01; ***p<0.001 versus control; $^{+}$p<0.05; $^{++}$p<0.001; $^{+++}$p<0.001 versus ChIS.*

4.3. Discussion

The study investigated the influence of the nitric oxide (NO) system on the analgesic effects of nociceptin N/OFQ(1-13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] in animals subjected to acute and chronic immobilization stress, using L-NAME (a NOS inhibitor) and L-arginine (a NO precursor).

Nitric oxide (NO) is a neurotransmitter involved in pain regulation at both central and peripheral levels. NO modulates the activity of the PAG (periaqueductal gray) and RVM (rostral ventromedial medulla), key structures in the descending inhibitory control of pain. Under stress, NO participates in the modulation of endogenous analgesic mechanisms, including interactions with opioid and NOP receptors. L-arginine and SIN-1 (a NO donor) induce antinociception mediated by the dynorphin system, highlighting the antinociceptive potential of NO under stress conditions.

Our results show that administration of L-arginine after acute stress increases the pain threshold in the PP test ($p<0.001$ at 10th and 20th min, $p<0.01$ at 30th min versus control; $p<0.05$ at 10th min and $p<0.001$ at 20th min versus 1hIS group) and prolongs latency in the HP test ($p<0.001$ versus control and 1hIS), indicating that L-arginine enhances stress-induced analgesia (SIA) (figs. 20 and 21).

Administration of L-NAME after stress significantly decreases the pain threshold ($p<0.001$ versus 1hIS) and shortens latency ($p<0.001$ versus 1hIS; $p<0.01$ at 10th min and $p<0.001$ at 20th and 30th min versus control), indicating that NOS inhibition reduces SIA (figs. 22 and 23). Co-administration of L-arginine with N/OFQ(1–13)NH_2 and its analogues significantly decreases the pain threshold ($p<0.001$, PP test) and shortens latency ($p<0.001$, HP test), reducing SIA. In the HP test, these changes are more pronounced, with latency reduced versus control ($p<0.001$), resulting in hyperalgesia (figs. 20 and 21).

Co-administration of L-NAME with N/OFQ(1–13)NH_2 and its analogues after stress also results in a significant decrease in pain threshold ($p<0.001$ versus 1hIS) and shortened latency ($p<0.001$ versus 1hIS), leading to suppression of SIA. In the HP test, latency is further reduced below control levels ($p<0.001$), inducing hyperalgesia (figs. 22 and 23).

Data from Xu et al. indicate that N/OFQ activates neuronal nitric oxide synthase (nNOS) via Ca^{2+} dependent mechanisms in the spinal cord, thereby stimulating local NO production. N/OFQ-induced pronociception is partially mediated through the NO/cGMP pathway – a mechanism that could explain the observed decrease in pain threshold and shortened latency.

The decrease in pain threshold and the shortening of latency observed with co-administration of N/OFQ(1-13)NH_2 and its analogues with L-arginine, as well as their co-administration with the NOS inhibitor after 1hIS, indicate that activation of NOP receptors under altered NO activity leads to hyperalgesia. This is consistent with the findings of Janicki & Jeske-Janicka and Cury et al., who described a pronociceptive role of NO during stress-induced iNOS activation. Additionally, Freire et al. emphasized that NOP receptors interact with NO/cGMP signaling, suggesting that disruption of this pathway through pharmacological intervention or stress-induced expression can shift NOP receptor activation from an analgesic to a pronociceptive effect. Under acute stress, as shown by Srebro et al., NMDA-mediated hyperalgesia is enhanced by NO, further explaining the observed results. Data from our previous studies indicate that N/OFQ(1–13)NH_2 and its analogues – [Orn^9] and [Orn^9, Orn^{13}] administered together with L-arginine or L-NAME after cold stress suppress SIA, significantly lowering the pain threshold in the PP test. Similar results have been reported for other opioid peptides (Tyr-MIF-1 analogues) applied after acute immobilization stress, which also reduced SIA, and whose effects were modulated by L-arginine and L-NAME.

Based on literature data and the results of our study, it can be concluded that nitric oxide participates in the modulation of SIA induced by acute immobilization stress. Increasing NO synthesis via L-arginine enhances SIA, whereas its inhibition via L-NAME reduces it. This indicates that NO is a key modulator of endogenous antinociceptive mechanisms activated under acute stress. Interaction of NO with nociceptin and its analogues after acute 1hIS reduces their analgesic effect, increasing pain sensitivity and suppressing SIA.

The results of the present study show that administration of L-arginine alone immediately after ChIS leads to a statistically significant increase in pain threshold compared to the control group ($p<0.001$) for the entire observation period and compared to the ChIS group ($p<0.01$ at 10 min; $p<0.001$ at 20 and 30 min). The highest threshold was recorded at the 20th minute in the PP test (192.3±6.9 g/cm²). In the HP test, a statistically significant prolongation of latency was observed compared to control ($p<0.001$) for the entire observation period and compared to ChIS ($p<0.001$ at 20 and 30 min). These results indicate that L-arginine induces analgesia under chronic stress (figs. 24 and 25). In the presence of L-arginine, N/OFQ(1–13)NH_2 and its analogues reduce the pain threshold and significantly shorten latency compared both to ChIS ($p<0.001$) and to the control group ($p<0.001$) at 10 and 20 minutes, resulting in hyperalgesia (figs. 24 and 25). Of particular interest are the peptide-specific and time-dependent effects observed with the co-administration of L-arginine with the analogues. In the PP test, an increase in pain threshold was recorded at the 20th minute for [Orn^9, Orn^{13}] and at the 30th minute for [Orn^9] ($p<0.01$ compared to control), which contrasts with the overall decrease in threshold. This suggests that structural modifications in the peptide sequence influence activity, duration of action, and interaction with NO.

In this context, the inclusion of ornithine in the peptide structure gains additional significance, given the metabolic relationship between ornithine and arginine. Ornithine is not a direct substrate for NOS but participates in the urea cycle, where it is in dynamic balance with arginine. Arginine is degraded to ornithine and urea via arginase, and ornithine can be used to regenerate arginine through citrulline synthesis, which is subsequently recycled back to arginine. This balance between ornithine and arginine is a key regulator of NO production. The study by Marini et al. shows that enteric arginase II provides ornithine for citrulline synthesis, highlighting the interconnection between these amino acids in nitrogen metabolism and NO signaling. Therefore, the analgesic effects observed with [Orn9] and [Orn9,Orn13] may result not only from the structural modification of the peptide but also from their influence on the local arginine/ornithine balance, which regulates NO production and, consequently, pain modulation.

Chronic stress induces biochemical and neurophysiological changes affecting pain sensitivity, including NOS regulation and modulation of the NOP

receptor system. NO can act as an antinociceptive mediator by activating NO/cGMP signaling, suppressing glutamatergic and substance P-mediated pathways, and enhancing the effects of endogenous and exogenous opioids. Simultaneously, NO interacts with NOP receptors, modulating G-protein-coupled pathways and secondary messengers such as cGMP. Under chronic stress, increased iNOS expression leads to NO overproduction and pronociceptive effects. This may explain the hyperalgesia observed with the administration of N/OFQ analogs under stress conditions.

Region-specific regulation of NOS isoforms, as described by Gądek-Michalska et al., suggests that the effects of NO on pain sensitivity are context-dependent, i.e., they depend on the type of stress, the site of action, and interactions with other neurotransmitters.

Under chronic stress, the interaction between the nitric oxide and nociceptin systems becomes particularly significant. Increased iNOS expression and NO overproduction can alter the effects of NOP receptor activation, leading to hyperalgesia or modulation of antinociceptive mechanisms. Co-administration of L-arginine with structurally modified N/OFQ(1–13)NH_2 analogues shows that NO can influence the duration and intensity of peptide-mediated effects, highlighting the role of NO as a key modulator of pain and the nociceptin system under stress.

Administration of L-NAME after ChIS significantly reduced the pain threshold in the PP test compared to the ChIS group throughout the study period ($p<0.001$), as well as compared to the control group at the 20th ($p<0.001$) and 30th minute ($p<0.05$). In the HP test, a significant shortening of latency was observed compared to the control group at the 10th minute ($p<0.001$) and at the 20th and 30th minutes ($p<0.01$), as well as compared to the ChIS group throughout the entire testing period ($p<0.001$). These results indicate that inhibition of NO synthesis leads to the loss of stress-induced antinociceptive effects and induces hyperalgesia (figs. 26 and 27).

Co-administration of L-NAME with N/OFQ(1–13)NH_2 and its analogues led to a significant reduction in pain threshold and shortening of latency compared to both the ChIS and control groups ($p<0.01$; $p<0.001$). The observed hyperalgesia suggests that inhibition of NO synthesis blocks the antinociceptive mechanisms activated by chronic stress and potentially enhances the pronociceptive effects of nociceptin peptides (figs. 26 and 27). An exception was observed in the L-NAME + [Orn^9] group, where at the 20th minute a significant increase in pain threshold was recorded compared to the control ($p<0.001$) and ChIS ($p<0.01$), which persisted at the 30th minute, although without statistical significance (fig. 26). This suggests that [Orn^9] may activate additional antinociceptive mechanisms independent of NO or influence the local metabolic balance between ornithine and arginine, potentially affecting other signaling systems.

The interaction between NO and the nociceptin system under chronic stress is complex and context-dependent. NOP receptors activated by N/OFQ can induce NO production via nNOS activation, particularly in the spinal cord. NO participates in antinociceptive mechanisms through cGMP-mediated signaling, which can potentially enhance the effects of NOP activation. Inhibition of NOS by L-NAME disrupts this signaling cascade, leading to the loss of the antinociceptive effect and enhanced hyperalgesia. This could explain the observed results with co-administration of L-NAME and N/OFQ analogs, where a reduction in pain threshold and shortening of latency were recorded.

5. Effects of N/OFQ(1–13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}] on serum levels of ACTH, cortisol, and adrenaline under acute and chronic immobilization stress

5.1. Serum ACTH levels

Acute immobilization stress significantly increases serum ACTH levels ($p<0.05$) compared to the control group (22.65 ± 2.15 pg/ml vs. 16.65 ± 2.35 pg/ml).

N/OFQ(1–13)NH_2 administered after stress reduces ACTH levels compared to the 1hIS group, but without statistical significance; ACTH levels remain elevated compared to control. The analogues [Orn^9] and [Orn^9, Orn^{13}] significantly decrease ACTH levels ($p<0.01$) compared to the 1hIS group.

Under chronic immobilization stress (ChIS), ACTH levels are comparable to the control (15.16 ± 1.96 pg/ml) and significantly lower ($p<0.01$) than in the 1hIS group.

For N/OFQ(1–13)NH_2, serum ACTH levels are similar to those in the ChIS group. In the [Orn^9] and [Orn^9, Orn^{13}] groups, an increase in ACTH levels compared to ChIS and control is observed, but without statistical significance (fig. 28).

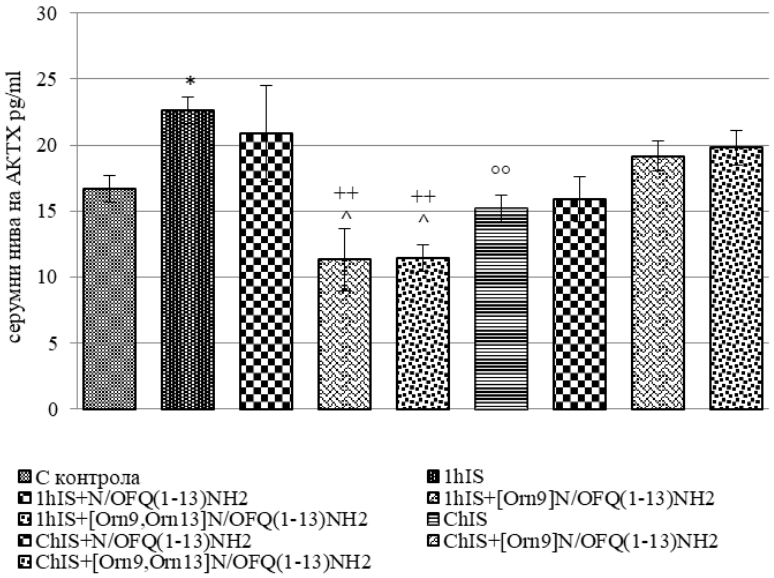

*Figure 28. Serum ACTH levels in animals subjected to 1hIS and ChIS and after administration of N/OFQ(1–13)NH₂ and analogues – [Orn⁹] and [Orn⁹, Orn¹³]. Data are presented as mean ± S.E.M.; *p<0.05 vs. control; ⁺⁺p<0.01 vs. 1hIS; °°p<0.01 between 1hIS and ChIS.*

5.2. Serum cortisol levels

1hIS significantly increases serum cortisol levels compared to the control group ($p<0.01$).

Administration of N/OFQ(1–13)NH_2 after 1hIS significantly decreases cortisol levels ($p<0.05$) compared to 1hIS (228.1 ± 22.7 ng/ml vs. 308.2 ± 6.07 ng/ml), suggesting an inhibitory effect of the peptide on HPA axis activation. The analogue [Orn^9] reduces cortisol levels compared to 1hIS and significantly increases them compared to the control group ($p<0.05$). The analogue [Orn^9, Orn^{13}] lowers cortisol levels relative to 1hIS, but the difference is not statistically significant. In ChIS, cortisol levels are significantly elevated compared to control ($p<0.01$; 300 ± 19.24 ng/ml vs. 177.36 ± 11.2 ng/ml).

After chronic stress, N/OFQ(1–13)NH_2 and its analogues decrease cortisol levels relative to ChIS, but without statistical significance. For [Orn^9], a significant increase in cortisol levels is observed compared to the control group ($p<0.01$) (fig. 29).

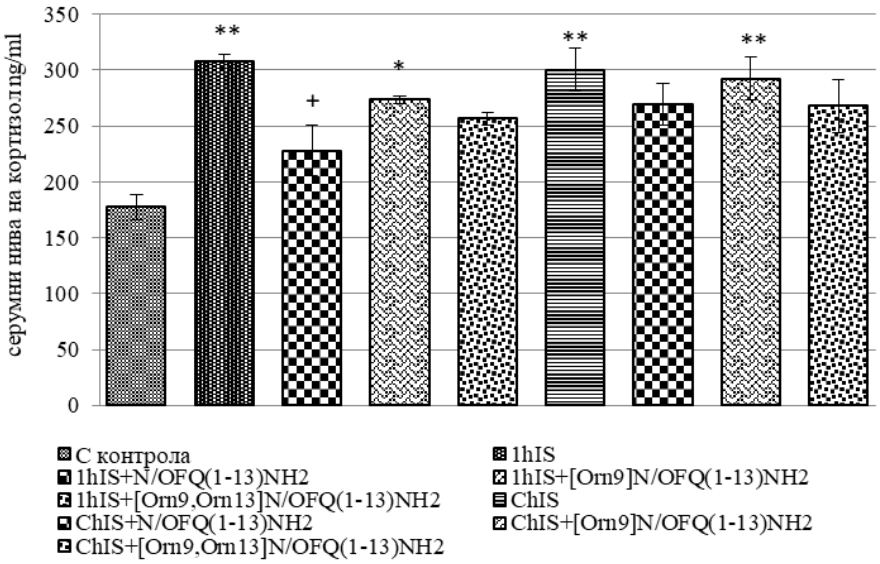

*Figure 29. Serum cortisol levels in animals subjected to 1hIS and ChIS, and after administration of N/OFQ(1–13)NH_2 and analogues – [Orn^9] and [Orn^9, Orn^{13}]. Data are presented as mean ± S.E.M.; *p<0.05, **p<0.01 vs. control; $^+$p<0.01 vs. 1hIS.*

5.3. Serum adrenaline levels

Acute immobilization stress (1hIS) significantly increases serum adrenaline levels compared to the control group ($p<0.01$; 1.56 ± 0.32 vs. 0.45 ± 0.07 ng/ml). Administration of N/OFQ(1–13)NH_2 after 1hIS further significantly elevates adrenaline levels compared to control ($p<0.01$). The analogues [Orn^9] and [Orn^9, Orn^{13}] reduce adrenaline levels relative to 1hIS, but without statistical significance; however, they remain higher than in the control group.

Chronic immobilization stress (ChIS) also significantly increases adrenaline levels compared to control ($p<0.05$; 0.97 ± 0.18 vs. 0.45 ± 0.07 ng/ml). Following ChIS, administration of N/OFQ(1–13)NH_2 significantly increases adrenaline levels compared to control ($p<0.01$), with values also being higher compared to the ChIS group. The analogue [Orn^9] decreases adrenaline relative to ChIS but remains elevated compared to control, whereas [Orn^9, Orn^{13}] increases adrenaline compared to control, with a response amplitude comparable to the ChIS group (fig. 30).

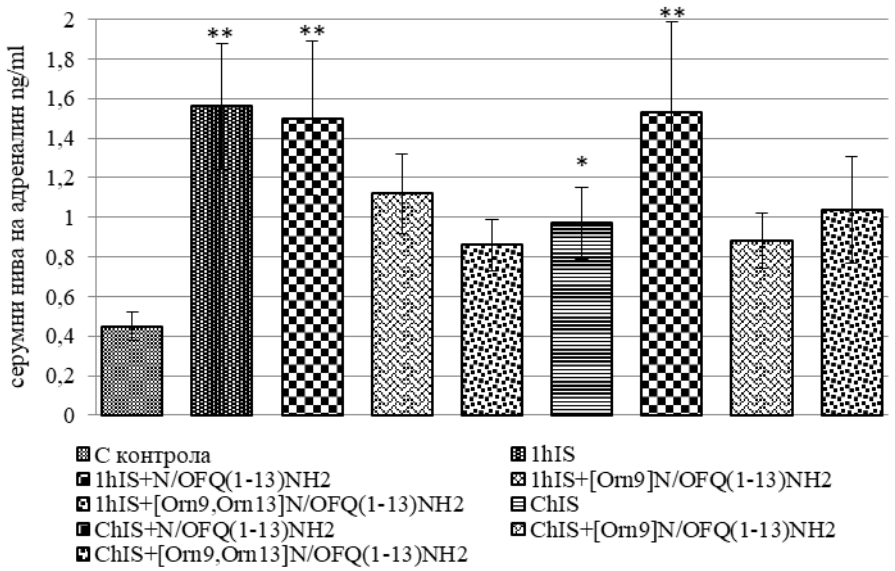

*Figure 30. Serum adrenaline levels in animals subjected to 1hIS and ChIS, and following administration of N/OFQ(1–13)NH₂ and analogues – [Orn⁹] and [Orn⁹, Orn¹³]. Data are presented as mean ± S.E.M.; *p<0.05, **p<0.01 versus control.*

5.4. Discussion

The nociceptin system plays a significant role in the regulation of the HPA axis and in the modulation of the neuroendocrine response to stress. Neurons expressing NOP receptors are localized in the hypothalamus, the amygdala, and other limbic structures, which actively participate in coordinating the hormonal response to stressful stimuli. Central activation of the NOP receptor stimulates the release of CRH and glucocorticoids, especially during acute stress, which leads to activation of the HPA axis and increased levels of ACTH and cortisol.

The results obtained by us show that 1hIS leads to a statistically significant increase in serum levels of ACTH ($p<0.05$), cortisol ($p<0.01$), and adrenaline ($p<0.01$) compared to the control, which is consistent with the well-documented activation of the SAM system and the HPA axis during stress. This increase reflects the physiological need for mobilization of energy resources and maintenance of homeostasis through catecholamine and glucocorticoid release during stress.

Serum ACTH levels show characteristic dynamics in response to 1hIS and ChIS, as well as after administration of N/OFQ(1-13)NH_2 and its analogues. In 1hIS, a statistically significant increase of ACTH is observed ($p<0.05$) compared to the control group (22.65±2.15 pg/ml vs 16.65±2.35 pg/ml), which confirms the

activation of the HPA axis and the CRH-secreting neurons in the PVN of the hypothalamus and the subsequent stimulation of the adenohypophysis.

Administration of N/OFQ(1-13)NH_2 after acute stress leads to a slight decrease in ACTH compared to 1hIS, suggesting a limited inhibitory effect of the peptide on hormonal secretion. In contrast, the analogues [Orn^9] and [Orn^9, Orn^{13}] induce a significant decrease in ACTH compared to 1hIS ($p<0.01$), showing a stronger inhibitory effect. This is probably due to increased affinity for the NOP receptor and improved biostability of the modified peptides.

During chronic stress, ACTH levels are reduced compared to 1hIS ($p<0.01$), which likely reflects adaptive mechanisms of the HPA axis and decreased sensitivity to prolonged stress exposure. Administration of [Orn^9] and [Orn^9, Orn^{13}] induces a slight increase in ACTH compared to ChIS, while with N/OFQ(1-13)NH_2 the values are comparable to those of ChIS (fig. 28).

In 1hIS, a statistically significant increase in serum cortisol levels is observed ($p<0.01$) compared to the control group (308.2±6.07 ng/ml vs 177.36±11.2 ng/ml), which confirms an endocrine response to an intense stress stimulus. This is consistent with literature data describing HPA activation with subsequent glucocorticoid secretion from the adrenal cortex. Administration of N/OFQ(1-13)NH_2 after acute stress leads to a statistically significant decrease in cortisol ($p<0.05$) compared to 1hIS (228.1±22.7 ng/ml vs 308.2±6.07 ng/ml), indicating an inhibitory effect of the peptide on HPA activation. This corresponds to the observations of Ubaldi et al., who described the role of the nociceptin system in limiting the hormonal response to stress via interaction with NOP receptors. The analogue [Orn^9] statistically significantly increases cortisol levels compared to the control ($p<0.05$), while decreasing them compared to 1hIS (273.7±3.32 ng/ml vs 308.2±6.07 ng/ml). [Orn^9, Orn^{13}] also decreases cortisol levels compared to 1hIS (256.4±5.5 ng/ml vs 308.2±6.07 ng/ml). These data indicate an inhibitory effect of the analogues on cortisol levels during acute stress, which is weaker compared to that of nociceptin.

In ChIS, a statistically significant increase in cortisol is observed ($p<0.01$) compared to the control (300±19.24 ng/ml vs 177.36±11.2 ng/ml), reflecting prolonged HPA axis activation and it's consistent with described adaptive mechanisms during prolonged stress exposure. Administration of N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}] leads to a decrease in cortisol levels compared to ChIS, but without statistical significance. The analogue [Orn^9] shows a statistically significant increase in cortisol levels compared to the control ($p<0.01$), with the response amplitude comparable to that of ChIS, suggesting that the inhibitory effect of the peptides is weaker during chronic stress (fig. 29).

The results show that the nociceptin system participates in the regulation of cortisol secretion, with the inhibitory effect of the peptides, especially N/OFQ(1-13)NH_2, being more pronounced during acute stress.

Our results show that both 1hIS and ChIS lead to a statistically significant increase in serum adrenaline levels ($p<0.01$) compared to the control, which is consistent with SAM activation during stress. Administration of N/OFQ(1-13)NH_2 after 1hIS and ChIS leads to a statistically significant increase in serum adrenaline levels ($p<0.01$) compared to the control. This observation is consistent with data showing that nociceptin may have a dual effect on the stress response, depending on the dose, time of administration, and type of stress. Of particular importance are the results with the administration of the analogues [Orn^9] and [Orn^9, Orn^{13}], which decrease adrenaline levels compared to 1hIS. This indicates that structural modifications at positions 9 and 13 enhance the anti-stressor activity of the peptide, likely through increased affinity for the NOP receptor or improved stability in the serum environment. The stronger inhibitory effect of [Orn^9, Orn^{13}] during acute stress suggests synergy between the two modifications in the peptide. With [Orn^9], adrenaline levels are higher than the control but lower than ChIS, while in ChIS + [Orn^9, Orn^{13}] an increase compared to the control is observed, with values close to ChIS. This indicates that [Orn^9] has a more pronounced inhibitory effect on sympathoadrenomedullary activation during chronic stress (fig. 30). The observed weakening of the inhibitory effect of [Orn^9, Orn^{13}] on adrenaline levels during ChIS can be explained by adaptive mechanisms of the HPA and SAM axes during prolonged stress exposure. According to McEwen, chronic stress leads to neuroendocrine adaptation, including changes in sensitivity to regulatory peptides, which may reduce their effect. Prolonged stress may induce desensitization or changes in NOP receptor expression, which could limit the ability of the analogs to exert their effect.

Additional support for the involvement of the nociceptin system in the regulation of the hormonal response to stress is provided by several experimental studies. According to Mallimo & Kusnecov, central administration of N/OFQ leads to activation of the HPA axis, while antagonism or genetic deficiency of the NOP receptor induces increased secretion of ACTH and corticosterone in response to stress. This suggests that the endogenous N/OFQ system plays a role in limiting the hormonal reaction to stress, maintaining neuroendocrine homeostasis.

Similar results have been reported by Leggett et al., who found that blocking the NOP receptor in models of inflammatory stress leads to elevated levels of ACTH and corticosterone. The authors hypothesize that the endogenous N/OFQ system functions as a modulator that limits excessive activation of the HPA axis under physiological or pathological stress conditions.

Delaney et al. demonstrate that central blockade of the NOP receptor leads to increased expression of CRF and POMC, as well as increased corticosterone secretion. These results confirm that the NOP receptor system actively participates in the modulation of the hormonal response during acute and chronic stress, affecting key components of the HPA axis.

Interestingly, NOP receptor antagonists, such as SB-612111, exhibit anxiolytic and antidepressant effects by suppressing the hormonal response to stress and reducing the secretion of CRH and glucocorticoids. This supports the hypothesis of the bidirectional effect of the nociceptin system: on one hand, it can enhance the response during acute stress, and on the other, it can limit excessive activation of the HPA axis during chronic or pathological conditions.

The observed effects of N/OFQ(1-13)NH_2 and its analogues on serum levels of ACTH, cortisol, and adrenaline in the present study support the hypothesis of the bidirectional role of the nociceptin system, either enhancing or limiting the hormonal response to stress depending on the type of stress, receptor expression, and structural characteristics of the ligand.

CONCLUSIONS

1. Nociceptin and its analogues – [Orn^9] and [Orn^9, Orn^{13}] statistically significantly increase the pain threshold and prolong latency time, thereby inducing analgesia in intact animals. The strongest analgesic effect is observed with the analogue [Orn^9, Orn^{13}].
2. Acute and chronic immobilization stress induce SIA, which is more pronounced in acute stress. Administration of nociceptin and its analogues after acute stress antagonizes SIA, decreasing the pain threshold and shortening latency time compared to the stress-exposed group, while under chronic stress these changes are observed relative to both the stress-exposed and control groups.
3. In intact animals, the selective antagonist JTC-801 increases the pain threshold and prolongs latency time compared to control. Pretreatment with JTC-801 significantly suppresses the analgesic effect of all tested peptides in the PP test, while in the HP test, blockade leads to hyperalgesia.
4. Co-administration of JTC-801 with the peptides leads to a statistically significant decrease in pain threshold and analgesia induced by both acute and chronic stress. Under acute stress, latency time is shortened with [Orn^9], while under chronic stress latency is shortened for all peptides compared to control, accompanied by the development of hyperalgesia.
5. In intact animals, naloxone statistically significantly decreases the pain threshold and shortens latency time compared to control, leading to hyperalgesia. Co-administration of naloxone with the peptides statistically significantly increases the pain threshold and prolongs latency time at the 10th minute compared to control, more pronouncedly with the analogues.
6. Under acute and chronic stress, co-administration of naloxone with the peptides statistically significantly decreases pain threshold and stress-induced analgesia and shortens latency time compared to control, causing hyperalgesia.
7. Co-administration of L-arginine with the peptides in intact animals statistically significantly increases the pain threshold and prolongs latency time compared to L-arginine alone, most pronounced at the 10th minute in both tests. Combination of L-NAME with the peptides significantly increases the pain threshold and latency time compared to L-NAME and control, with response amplitudes lower than those observed with L-arginine.

8. Under acute and chronic stress, L-arginine statistically significantly increases the pain threshold and prolongs latency time compared to the stress-exposed group. Co-administration of L-arginine with nociceptin and its analogues under acute and chronic stress reduces SIA (decreases pain threshold) and shortens latency time compared to control in the HP test (causing hyperalgesia).
9. Under acute stress, L-NAME reduces SIA (decreases pain threshold) and shortens latency time compared to control in the HP test (causing hyperalgesia). Under chronic stress, L-NAME induces hyperalgesia in both tests, statistically significantly decreasing pain threshold and shortening latency time compared to control.
10. Under acute stress, co-administration of L-NAME with the peptides reduces SIA (decreases pain threshold) and shortens latency time compared to control in the HP test (causing hyperalgesia), while under chronic stress hyperalgesia is observed in both nociceptive tests. The exception is the analogue [Orn^9], which at the 20th minute statistically significantly increases the pain threshold compared to control and the chronic stress group.
11. Acute stress increases serum levels of ACTH, cortisol, and adrenaline compared to control, while chronic stress leads to increased cortisol and adrenaline without significant changes in ACTH compared to control. Compared to acute stress, chronic stress is characterized by lower levels of ACTH and adrenaline and comparable cortisol concentrations.
12. Under acute stress, the analogues statistically significantly decrease serum ACTH levels compared to control and the stress-exposed group, while under chronic stress they increase ACTH levels. In both stress models, the peptides decrease cortisol levels.
13. Under acute and chronic stress, nociceptin statistically significantly increases serum adrenaline levels compared to the control group. The analogues decrease adrenaline levels under acute stress.

CONTRIBUTIONS

Original Contributions

1. The analgesic effects of newly synthesized nociceptin analogues N/OFQ(1-13)NH_2 – [Orn^9]N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 were compared in intact animals.
2. For the first time, the effects of the newly synthesized nociceptin analogues N/OFQ(1-13)NH_2 – [Orn^9]N/OFQ(1-13)NH_2 and [Orn^9, Orn^{13}]N/OFQ(1-13)NH_2 on stress-induced analgesia (SIA) elicited by acute and chronic immobilization stress were investigated.
3. For the first time, the combined effects of JTC-801 with nociceptin or with the newly synthesized analogues were examined in intact animals and on SIA induced by acute and chronic immobilization stress.
4. For the first time, the combined effects of naloxone with nociceptin or with the newly synthesized analogues were studied in intact animals and on SIA induced by acute and chronic immobilization stress.
5. For the first time, the combined effects of L-arginine with nociceptin or with the newly synthesized analogues were investigated in intact animals and on SIA induced by acute and chronic immobilization stress.
6. For the first time, the combined effects of L-NAME with nociceptin or with the newly synthesized analogues were examined in intact animals and on SIA induced by acute and chronic immobilization stress.
7. For the first time, the effects of nociceptin and the newly synthesized analogues on serum levels of ACTH, cortisol, and adrenaline under acute and chronic immobilization stress were studied.

LIST OF PROJECTS, PUBLICATIONS, AND PARTICIPATIONS RELATED TO THE DISSERTATION

Publications and reports in scientific journals indexed in internationally recognized databases (Scopus and Web of Science):

1. **Himcheva I,** Stavreva GT, Naydenova E, Bocheva A. *Involvement of the opioidergic and nociceptinergic systems in the analgesic effects of novel nociceptin analogues after acute and chronic immobilization stress.* Pharmacia. 2022 Jun 10;69(4):935-42. ISSN: 0428-0296. SJR 0.212, IF 1.1. Indexed in Scopus and Web of Science.

Publications in scientific journals not indexed in Scopus or Web of Science, including full-text publications (with book or conference abstracts):

1. **Himcheva I**, Stavreva G, Angelova N, Naydenova E, Krastev D, Kochev D, Bocheva A. Involvement of the Opioidergic System in the Analgesic Effects of Newly Synthesized Nociceptin Analogs in Immobilization Stress. Health and Science. 2020, Issues 1–2, pp. 41–44. ISSN 1314-3360.

2. **Himcheva I**, Stavreva G, Simeonova T, Angelova N, Naydenova E, Krastev D, Kochev D, Bocheva A. Effects of Nociceptin and Analogs on Pain Perception after Heat Stress in Rats. Health and Science. 2020, Issues 1–2, pp. 36–40. ISSN 1314-3360.

3. Bocheva A, Kastelova A, **Himcheva I**, Dimitrova A, Grigoryan A, Krastev N, Kalniev M, Krastev D. Biologically Active Peptides: Concepts. Health and Science. 2022, Issues 3–4, pp. 18–22. ISSN 1314-3360.

4. Krastev D, Kastelova A, Dimitrova A, Grigoryan A, Krastev N, Kalniev M, **Himcheva I,** Bocheva A. Neuropeptides: Their Neuromodulatory Role in the CNS under Normal Conditions. Health and Science. 2022, Issues 3–4, pp. 28–31. ISSN 1314-3360.

Participation in scientific events with oral/poster presentations and published abstracts in conference proceedings/journals:

1. **Himcheva I**, Nocheva HH, Pavlov ND, Naydenova ED, Bocheva AI. Involvement of Naloxone and JTC-801 in Analgesic Effects of Newly Synthesized Nociceptive Analogs. JBCR. Vol.12 Number 1,Supplement 2, 2019,127-128

2. **Himcheva I**, Angelova NG, Bocheva AI, Naydenova ED. Analgesic Effects and Synthesis of New Analogues of Nociceptin. JBCR. Vol.12 Number 1,Supplement 2, 2019,128
3. Krastev PK, **Himcheva I**, Dzhambazova EB, Stavreva GT, Lakova ET,Bocheva AI. Effects of Leu-Enkephalin Analogue on Stress-Induced Analgesia in Rats. JBCR. Vol.12 Number 1,Supplement 2, 2019,123-124
4. N. Angelova, **I. Himcheva**, A. Bocheva, E. Naydenova. Synthesis and Biological Activity of Nociceptin/OFQ(1–13)NH_2 Analogues. XVI Scientific Poster Session for Young Scientists, PhD Students and Students, 100 Years of the International Union of Pure and Applied Chemistry, Book of Abstracts, UCTM – 23 May 2019, VII-10, p. 124.
5. N. Angelova**, I. Himcheva**, A. Bocheva, E. Naydenova. Synthesis and Biological Activity of Nociceptin/OFQ(1–13)NH_2 Analogues. VI Scientific Session for Students, PhD Students and Young Scientists, UCTM – 7 November 2019, Sofia.
6. **Himcheva I**, Stavreva G, Grigoryan A, Dimitrova A, Bocheva A. Participation of Nitric Oxide in the Analgesic Effects of Newly Synthesized Nociceptin Analogs after Chronic Immobilization Stress. Jubilee Scientific Conference "60 Years of Pathophysiology" – Medical University–Varna, 24–25 September 2021 (online).
7. **Himcheva I**., G. Stavreva, N. Angelova, E. Naydenova, A. Bocheva . Involvement of the nitric oxydeergic system in the analgesic effects of newly synthesized nociceptin analogues after chronic immobilization stress. The EuroBiotech Journal DOI: 10.2478/ebtj-2021-0031, European Biotechnology Congress September 23-25, 2021 in Sofia, Bulgaria.
8. **Ivelina Himcheva**, Emilia Naydenova, Galiya Stavreva, Adriana Bocheva. Participation of the opioidergic and nociceptinergic systems in the effects of nociceptin and new analogues after acute and chronic immobilization stress. International Scientific Conference of the Bulgarian Peptide Society, e-Abstract book 2021; 46-48, 9th International Conference of Peptides (hybride), Velingrad, Bulgaria, 27.08 – 29.08.2021
9. **Himcheva I.**, Angelova N., Stavreva G., Naydenova E., Bocheva A. Nociceptin and New Analogs: Effects and Mechanisms on Stress-Induced Analgesia. National Meeting for "Building a Scientific Research Network," National Programme "European Scientific Networks," "Drug-Molecule," e-book 2021; pp. 227–251, Sofia, Bulgaria, 30.06.2021.

10. **Ivelina Himcheva,** Adriana Bocheva. Biological activity of some newly synthesized analogues of nociception. Ежегоден конкурс „Наука и Младост – 2022“, 08.04.-10.04.2022 МУ – Пловдив
11. **Ivelina Himcheva**, Galya Stavreva, Tatyana Simeonova, Anelia Dimitrova, Emilia Naydenova, Adriana Bocheva. Nociceptin and analogues: Involvement of the cannabinoidergic system on pain after chronic immobilization stress. national conference with international participation “Innovations in drug molecules”19-22 July, 2022; Hisarya, Bulgaria. P78-80.ISBN: 978-954-465-142-8
12. **Ivelina Himcheva**, Borislav Ignatov, Antonia Kastelova, Adriana Bocheva. Nociceptin and its role in opioid regulation in the brain, 12th SouthEast European Conference and 32st Annual Assembly of IMAB 20-23 October 2022 Trakia University of Stara Zagora, Bulgaria
13. Ademira Borisova, **Ivelina Himcheva**, Armine Grigoryan, Anelia Dimitrova, Adriana Bocheva. Are catecholaminergic neurotransmission and nociceptin analogues involved in chronic stress?, 12th South-East European Conference and 32st Annual Assembly of IMAB 20-23 October 2022 Trakia University of Stara Zagora, Bulgaria
14. **Ivelina Himcheva**, Galya Stavreva, Adriana Bocheva Role of nociceptin and analogues in the interaction between nitricoxidergic and endocannabinoidergic systems after chronic immobilization stress in rats, International Conference of the Bulgarian Society of Physiological Sciences; 2022 Oct 30 – Nov 1; Stara Zagora Mineral Springs, Bulgaria. Sofia: Elestra EOOD; 2022. p. 63-4. ISBN: 978-619-7292-23-7.
15. **Himcheva I.,** G. Stavreva, A. Grigorian, A. Dimitrova, E. Naydenova, A. Bocheva Effects of nociceptin and analogues and the involvement of the nitricoxidergic system on nociception after chronic immobilization stress, International Conference of the Bulgarian Society of Physiological Sciences; 2022 Oct 30 – Nov 1; Stara Zagora Mineral Springs, Bulgaria. Sofia: Elestra EOOD; 2022. p. 70-1. ISBN: 978-619-7292-23-7.
16. **Himcheva I**., G. Stavreva, A. Borisova, A. Grigorian, A. Dimitrova, E. Naydenova, A. Bocheva, Effects of nociceptin and analogues on the cannabinoidergic system on pain after chronic immobilization stress, International Conference of the Bulgarian Society of

Physiological Sciences; 2022 Oct 30 – Nov 1; Stara Zagora Mineral Springs, Bulgaria. Sofia: Elestra EOOD; 2022. p. 66-7. ISBN: 978-619-7292-23-7.

17. **Himcheva I**., T. Simeonova, E. Naydenova, G. Stavreva. Involvement of the opioid and endocannabinoid systems in the analgesic effects of novel nociceptin analogues after chronic immobilisation stress. Jubilee Scientific Conference "50 Years of Medical Education and Science in Pleven" 1-3.11.2024. Abstract book: p 184. ISBN: 978-954-756-347-6
18. **Himcheva I.,** A. Borisova, A. Grigorian, G. Stavreva, A. Dimitrova. Effects of novel nociceptin analogues on the adrenergic and opioidergic systems after chronic immobilisation stress. Jubilee Scientific Conference "50 Years of Medical Education and Science in Pleven" 1-3.11.2024. Abstract book: p 183. ISBN: 978-954-756-347-6
19. Galia Stavreva, **I. Khimcheva**, E. Naydenova. Analogs of nociceptin and their effects on the endocannabinoid and opioid systems after chronic immobilization in rats. VIII National Congress on Pharmacology, Clinical Pharmacology and Therapy. Pharmacology from Experiment to Clinic. 15–17 November 2024, Medical University–Pleven.

Participation in Research Projects:

1. "Nociceptin and Newly Synthesized Analogs: Effects and Mechanisms of Action on Nociception in a Chronic Stress Model," Department of Physiology and Pathophysiology, Medical University–Pleven, Project No. 10/2020
2. "The Biological Activity of Several Newly Synthesized Analogs of Nociceptin," Department of Physiology and Pathophysiology, Medical University–Pleven, Project No. 19/2019
3. "Effects of the Endocannabinoid and Nitric Oxide Systems on Pain Perception and Acute Inflammatory Pain in Cold Stress-Induced Analgesia in Rats," Department of Pathophysiology, Medical University–Sofia, Project No. 42/2014