

STUDY OF EFFECTS OF NEWLY SYNTHESISED NOCICEPTIN ANALOGUES ON THE ENDOCANNABINOID SYSTEM AND PAIN AFTER CHRONIC IMMOBILIZATION STRESS

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ABSTRACT

Stress provokes stress-induced analgesia (SIA), which depends on an opioid and non-opioid components. The non-opioid one comprises several systems among which are endocannabinoid (ECS), adrenergic, and nitricoxidergic participating in the descending antinociceptive system of the body. The ECS system has a well-established role in the modulation of pain perception and behavioral responses after stress. Nociceptin/Orphanin FQ(N/OFQ) is a heptadecapeptide that has been found to play a role in pain perception. This study aimed to investigate the effects of novel nociceptin N/OFQ(1-13)NH₂ analogues on nociception after chronic immobilization stress (CIS) and the involvement of the ECS in analgesic effects. The experiments were carried out on male Wistar rats. The animals were immobilized in a tube for 3 hours daily for 4 days. Analgesic effects were examined by the paw-pressure (PP) test. All novel analogues of N/OFQ(1-13)NH₂, the cannabinoid receptor type 1 (CB1-receptor) agonist N-arachidonylethanolamide (AEA), and the CB1-receptors antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) were administered intraperitoneally (i.p) dissolved in Dimethyl sulfoxide (DMSO). Statistical analysis was performed using one-way ANOVA. The results showed that nociceptin and analogues administered after CIS decreased the pain threshold significantly compared to a group that underwent chronic stress only. The administration of AEA immediately after the end of stress led to a significantly increased pain threshold, while administration of AM251 significantly decreased the pain threshold versus the both control and group that underwent chronic stress only. Nociceptin and analogues co-administered with CB1-receptor agonist (AEA) or antagonist (AM251) after the end of stress decreased immobilization SIA. Our study gives us reason to assume the participation of ECS in the analgesic effects of the novel nociceptin analogues after chronic immobilization stress.

Keywords: nociceptin analogues, endocannabinoid system (ECS), pain, chronic stress.

INTRODUCTION

Stress has an influence on the central nervous system (CNS), endocrine, and immune systems and evokes functional and structural changes in the body [1]. It is known that stress elicits pain alleviation, a phenomenon referred to as stress-induced analgesia

(SIA). Two forms of SIA are commonly distinguished: an opioid-mediated and a non-opioid one [2, 3]. The non-opioid one comprises cannabinoidergic, adrenergic, and nitricoxidergic systems, participating in the descending antinociceptive system of the body [4 - 6]. In recent years, there has been growing evidence that the ECS

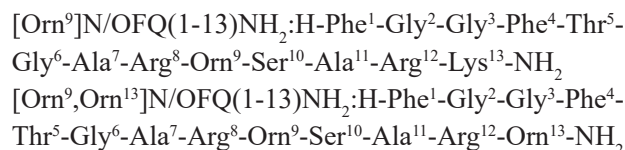
has a regulatory role in the processing and perception of pain and in the modulation of behavioral responses after stress [1, 3, 7]. The ECS consists of two main G-protein-coupled receptors, cannabinoid 1 receptor (CB1) and cannabinoid 2 receptor (CB2) [8 - 10], as well as their natural ligands N-arachidonylethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) [11 - 13], which are the most well-studied endocannabinoids, and the enzymes responsible for their synthesis and degradation. CB1 receptors are localized in brain regions involved in the modulation of pain including the rostral ventromedial medulla (RVM), the periaqueductal gray (PAG), amygdala, and prefrontal cortex (PFC) with these brain regions also key components of stress [14 - 16]. CB2 receptors are found principally in the immune system and to a lesser extent in the CNS [17]. Activation of cannabinoid (CB) receptors on presynaptic nerve terminals usually function to decrease neurotransmission, resulting primarily in antinociception/analgesia [18, 19]. Endocannabinoids (eCBs) decrease nociception by suppression of cAMP synthesis and activation of Gi-proteins [20].

Nociceptin/orphanin FQ(N/OFQ) is an endogenous 17 amino acid neuropeptide with a wide distribution in the CNS [21, 22]. N/OFQ is derived from pronociceptin/orphanin FQ [23, 24]. The amino acid sequence of nociceptin is very similar to that of other opioid peptides, especially Dynorphin A, which is evidence of the close evolutionary link between precursors. N/OFQ modulates a number of physiological functions including nociception, learning and memory, and motivated behaviors [23, 25 - 27]. These effects have been attributed to activation of the opioid receptor-like 1 (ORL-1; also known as NOP) receptor, a novel member of the opioid receptor family, which has a high level of homology to the classical mu (μ), delta (δ), and kappa (κ) receptors [23]. N/OFQ has anti-opioid effects [23, 24] which are antagonized by naloxone [28, 29] and influenced the development of morphine sensitization and tolerance in experimental animals [30, 31].

Increasing evidence suggests that stress modulates endogenous N/OFQergic signaling. This includes evidence regarding the distribution of the peptide N/OFQ and the NOP receptor protein in brain regions important in stress [32, 33].

The purpose of the present study was to investigate the influence of the amino acid Lys⁹ and Lys¹³ in new

analogues of N/OFQ(1-13)NH₂ (H-Phe¹-Gly²-Gly³-Phe⁴-Thr⁵-Gly⁶-Ala⁷-Arg⁸-Lys⁹-Ser¹⁰-Ala¹¹-Arg¹²-Lys¹³-NH₂) on nociception after the end of chronic immobilization stress (CIS) alone and in combination with a CB1-receptor agonist (AEA) or antagonist (AM251). For this purpose, the following peptides were synthesized by Solid Phase Peptide Synthesis, Fmoc-strategy according to Naydenova et al. [34]:



EXPERIMENTAL

Chemistry

The protected amino acids and Fmoc-Rink Amide MBHA Resin were purchased from Iris Biotech (Germany). All other reagents and solvents were analytical or HPLC grade and were bought from Merck (Germany). The LC/MC spectra were recorded on a LTQ XL Orbitrap Discovery instrument, Thermo Corporation, USA. The optical rotation was measured on automatic standard polarimeter Polamat A, Carl Zeiss, Jena. The conventional solid-phase peptide synthesis based on Fmoc (9-fluorenylmethoxycarbonyl) chemistry was employed to synthesize a series of new analogues of N/OFQ (1-13). Rink-amide MBHA resin and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) or N,N'-Diisopropylcarbodiimide (DIC) were used as solid-phase carrier and condensing reagent. Three-functional amino acids were embedded as N^α-Fmoc-Thr(tBu)-OH, N^α-Fmoc-Lys(Boc)-OH, N^α-Fmoc-Orn(Boc)-OH, N^α-Fmoc-Arg(Pbf)-OH. The coupling reactions were performed, using for amino acid/TBTU/HOBt/DIEA/resin a molar ratio 3/3/3/9/1 or amino acid/DIC/HOBt/resin a molar ratio 3/3/3/1. The Fmoc-group was deprotected by a 20 % piperidine solution in N,N-Dimethylformamide. The coupling and deprotection reactions were checked by the Kaiser test. The cleavage of the synthesized peptide from the resin was done, using a mixture of 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilane (TIS) and 2.5 % water. The peptide was obtained as a filtrate in TFA and precipitated with cold dry ether. The precipitate was filtered, dissolved in water and lyophilized to obtain the crude peptide. The

peptide purity was monitored on a RP-HPLC XTera C18 3.5 μm (125 x 2.1 mm) (Waters Co.) column, flow 200 $\mu\text{l}/\text{min}$, using a linear binary gradient of phase B from 10 % to 90 % for 15 min (phase A: 0.1 % $\text{HCOOH}/\text{H}_2\text{O}$; phase B: 0.1 % HCOOH/AcCN). The compounds were checked by electrospray ionization mass spectrometry and the optical rotation was measured in water.

Biological studies

The experiments were carried out on 78 male Wistar rats (180-200g) kept under normal conditions at ambient room temperature ($22 \pm 2^\circ\text{C}$), maintained under a 12 h/12 h light/dark regime, and supplied with standard chow and water ad libitum. The animals were divided into 13 groups, each one consisted of 6 animals. All experiments were performed in accordance with the requirements of the Bulgarian Food Safety Agency for work with animals with a registration license № 239.

Chronic model of immobilization stress

The animals are placed for 3 hours daily for 4 days in special transparent plastic cylinders with breathing holes, limiting their movements to a minimum.

Nociceptive test

The evaluation of antinociceptive effects was carried out using the paw-pressure (PP) test [35]. The changes in the mechanical nociceptive thresholds of the rats were measured by an analgesimeter (Ugo Basile). The pressure was applied to the rat hind-paw and the pressure (g) required for eliciting a nociceptive response, such as a squeak or struggle, was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was observed to prevent damage of the paw.

Drugs and treatment

Nociceptin analogues were synthesized in the laboratory of Prof. Ph.D. E. Naydenova in the University of Chemical Technology and Metallurgy, Sofia. All novel analogues of N/OFQ were injected i.p. immediately after stress at a dose of 10 $\mu\text{g kg}^{-1}$. The cannabinoid receptor type 1 (CB1-receptor) agonist N-arachidonylethanolamide (AEA, 1 mg kg^{-1}) and the CB1-receptors antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251, 1,25 mg kg^{-1}) were applied intraperitoneally (i.p) immediately after the

end of stress and 10 min before peptides, dissolved in Dimethyl sulfoxide (DMSO). The control group was not submitted to stress procedure and was injected with saline 0.1 ml kg^{-1} , i.p.

Data analysis

The results were presented as mean values \pm S.E.M. and were tested by one-way ANOVA, followed by Fisher's least significant difference procedure as a post-hoc test. The differences between the groups were considered statistically significant at $p \leq 0.05$. Analyses were performed using STATGRAPHICS® Centurion XV statistical software.

RESULTS AND DISCUSSION

In order to study and establish the influence of the amino acids Lys⁹ and Lys¹³, we synthesised by Solid Phase Peptide Synthesis, Fmoc-strategy, the following new fragment analogs of N/OFQ(1-13)NH₂ (H-Phe¹-Gly²-Gly³-Phe⁴-Thr⁵-Gly⁶-Ala⁷-Arg⁸-Lys⁹-Ser¹⁰-Ala¹¹-Arg¹²-Lys¹³-NH₂):

[Orn⁹]N/OFQ(1-13)NH₂:H-Phe¹-Gly²-Gly³-Phe⁴-Thr⁵-Gly⁶-Ala⁷-Arg⁸-Orn⁹-Ser¹⁰-Ala¹¹-Arg¹²-Lys¹³-NH₂

[Orn⁹,Orn¹³]N/OFQ(1-13)NH₂:H-Phe¹-Gly²-Gly³-Phe⁴-Thr⁵-Gly⁶-Ala⁷-Arg⁸-Orn⁹-Ser¹⁰-Ala¹¹-Arg¹²-Orn¹³-NH₂

The compounds were tested on nociception after the end of chronic immobilization stress (CIS) alone and in combination with a CB1-receptor agonist (AEA) or antagonist (AM251).

The experimental studies were performed following several protocols:

1) In the first place the impact of chronic immobilization stress (CIS) on nociception was estimated and the effects of the application of N/OFQ and the novel analogues immediately after the end of stress.

Our results showed that CIS increased the pain threshold of the experimental animals on the 10th min only, which was statistically significant ($p < 0.05$) versus the controls. On the 20th and 30th min, CIS caused a slight increase in pain threshold, which was not statistically significant compared to controls. Nociceptin and analogues [Orn⁹]N/OFQ(1-13)NH₂, [Orn⁹,Orn¹³]N/OFQ(1-13)NH₂ administered after CIS decreased the pain threshold significantly ($p < 0.05$) compared to a group that underwent chronic stress only. The newly synthesised analogue of N/OFQ(1-13)NH₂, in which

the Lys at the 9th and 13th positions substituted with Orn suppressed the pain threshold more strongly for the whole period of the study than that of $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$ after the end of chronic immobilization (Fig. 1).

Literature data showed that immobilization stress causes an increase in antinociception in tail-flick [36], hot-plate [37], and formalin tests [38]. The evidence that N/OFQ decreased tail-flick and hotplate latency, when centrally administered, was reported with the discovery of this peptide [39]. Literature and previous data of ours showed that the administration of the amino acid L-ornithine intracerebroventricularly (i.c.v.) elicited significant antinociception in the mechanical and thermal nociceptive tests in intact mice [40, 41]. Analogues with Orn instead of Lys at the 13th position have a more pronounced analgesic effect, while the analogues with Orn at the 9th position have a significantly less pronounced analgesic effect, compared to N/OFQ(1-13) NH_2 [40, 42].

2) The participation of the ECS in the analgesic effects of the novel nociceptin analogues was confirmed by AEA-administration immediately after the end of stress (CIS) and with the combinations of AEA+N/OFQ(1-13) NH_2 , AEA+ $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$, and AEA+ $[\text{Orn}^9, \text{Orn}^{13}]\text{N}/\text{OFQ}(1-13)\text{NH}_2$.

Anandamide (AEA, 1 mg kg^{-1} , i.p.) was injected immediately after the end of stress and 10 min before

investigated nociceptin analogues. The administration of CB1-receptor agonist (AEA) immediately after the end of stress led to a significantly ($p < 0.05$) increased pain threshold of the experimental animals during the whole period of the study compared to the control group and the group that underwent chronic stress only. Co-administration of AEA with nociceptin (AEA+N/OFQ(1-13) NH_2) and AEA with analogues (AEA+ $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$, AEA+ $[\text{Orn}^9, \text{Orn}^{13}]\text{N}/\text{OFQ}(1-13)\text{NH}_2$) immediately after the end of the stress decreased significantly ($p < 0.05$) pain threshold, compared to a group that underwent chronic stress and group administered with AEA only (Fig. 2).

3) The participation of the ECS in the analgesic effects of the novel nociceptin analogues was confirmed by AM251 pretreatment applied immediately after the end of stress (CIS) and with the combination of AM251+N/OFQ(1-13) NH_2 , AM251+ $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$, AM251+ $[\text{Orn}^9, \text{Orn}^{13}]\text{N}/\text{OFQ}(1-13)\text{NH}_2$.

Application of CB1-receptor antagonist (AM251, 1,25 mg kg^{-1} , i.p.) immediately after the end of chronic immobilization significantly reduced ($p < 0.05$) the pain threshold during the whole period of the study compared to the group that underwent only chronic stress and the control group. Nociceptin and analogues $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$, $[\text{Orn}^9, \text{Orn}^{13}]\text{N}/\text{OFQ}(1-13)\text{NH}_2$ co-administered with AM251 after the end of stress

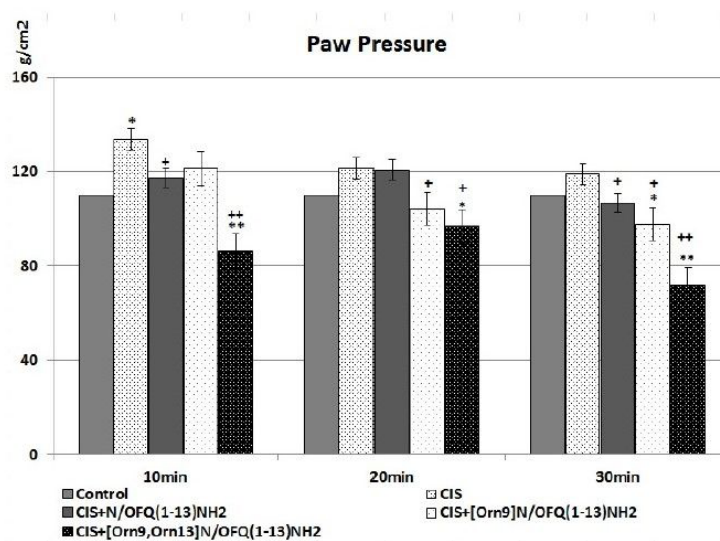


Fig.1. Effects of nociceptin N/OFQ(1-13) NH_2 and novel nociceptin analogues $[\text{Orn}^9]\text{N}/\text{OFQ}(1-13)\text{NH}_2$, $[\text{Orn}^9, \text{Orn}^{13}]\text{N}/\text{OFQ}(1-13)\text{NH}_2$ (all at a dose 10 $\mu\text{g kg}^{-1}$, i.p.) on nociception measured by Paw-Pressure (PP) test in male Wistar rats after stress (CIS). Mean values \pm S.E.M. are presented (* $p < 0.05$; ** $p < 0.01$ vs. control; + $p < 0.05$; ++ $p < 0.01$ vs. CIS).

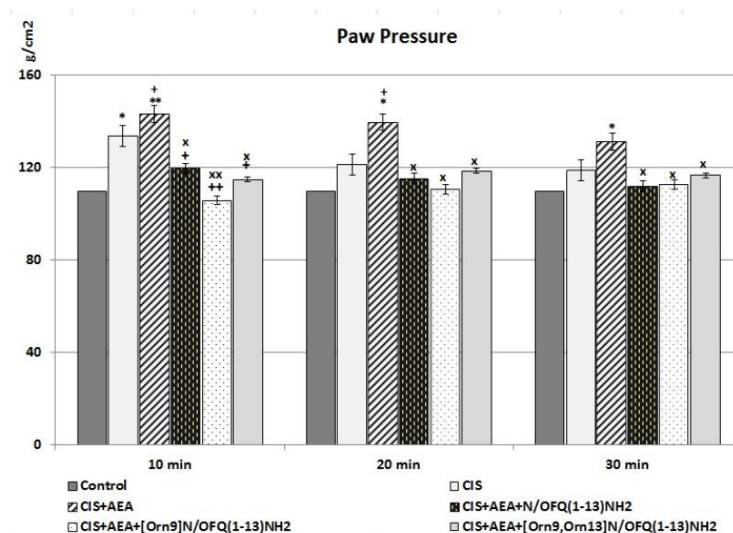


Fig. 2. Effects of anandamide (AEA, 1 mg kg⁻¹, i.p) co-administration with N/OFQ(1-13)NH₂ and novel analogues [Orn⁹]N/OFQ(1-13)NH₂, [Orn⁹,Orn¹³]N/OFQ(1-13)NH₂ (all at a dose 10 µg kg⁻¹, i.p) on the pain threshold (PP) test in male Wistar rats after stress (CIS). Mean values ± S.E.M. are presented (*p < 0.05; **p < 0.01 vs. control; +p < 0.05; ++p < 0.01 vs. CIS; x p < 0.05; xx p < 0.01 vs. CIS+AEA).

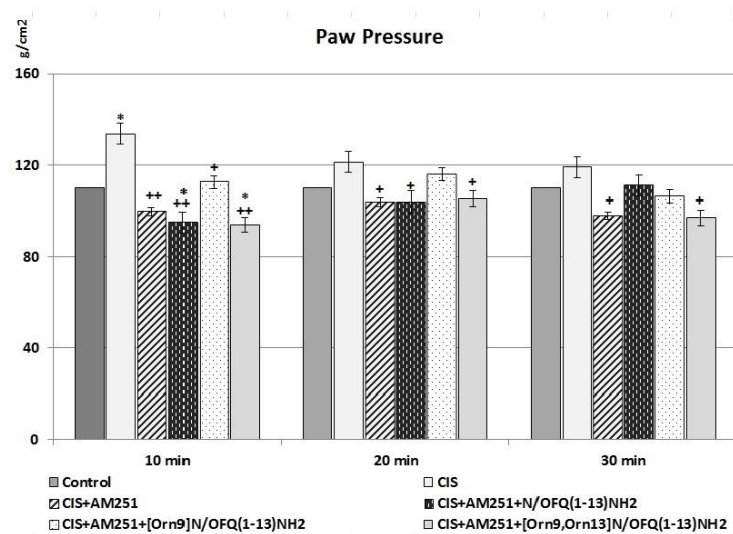


Fig. 3. Effects of AM251 (CB1 antagonist at a dose of 1,25 mg kg⁻¹) co-administration with N/OFQ(1-13)NH₂ and novel analogues [Orn⁹]N/OFQ(1-13)NH₂, [Orn⁹,Orn¹³]N/OFQ(1-13)NH₂ (all at a dose 10 µg kg⁻¹, i.p) on the pain threshold (PP) test in male Wistar rats after stress (CIS). Mean values ± S.E.M. are presented (*p < 0.05 vs. control; +p < 0.05; ++p < 0.01 vs. CIS).

decreased the pain threshold significantly ($p < 0.05$) on the 10th, 20th and 30th min compared to the group with CIS only. PP-thresholds of the animals from both experimental groups CIS+AM251+N/OFQ(1-13)NH₂ and CIS+AM251+[Orn⁹,Orn¹³]N/OFQ(1-13)NH₂ were comparable to the group CIS+AM251 on the 10th and 20th min, while the pain threshold was higher in the

group CIS+AM251+ [Orn⁹]N/OFQ(1-13)NH₂ than aforementioned groups (Fig. 3).

The obtained results give us reason to assume that chronic immobilization stress induces mild hypoalgesic effects, in which the pain perception is mediated by the cannabinoidergic neurotransmitter system. Activation of CB1-receptors by exogenous CB1-receptor agonist

(AEA) increases immobilization SIA, while inhibition of the CB1-receptor by antagonist AM251 decreased SIA after chronic immobilization. It is generally accepted that the analgesic activity of cannabinoids is mediated through the activation of CB1 receptors, which are expressed in areas involved in nociception [43 - 46]. Cannabinoid-induced antinociception appears mostly at spinal and supraspinal sites, but a peripheral action has also been suggested [44, 47]. In vivo experiments showed the transient increase in the content of two CB1-receptor agonists-anandamide and 2-arachidonoylglycerol in the periaqueductal gray (PAG) after foot-shock stress [48]. There is substantial evidence that an increase in endocannabinoid signaling contributes to non-opioid stress-induced analgesia [49]. The initial demonstration of this effect found that CB1 receptor-deficient mice did not exhibit antinociception following exposure to swim stress [50] and that global disruption of CB1 receptor signaling blocks stress-induced analgesia [51].

It is known that stress affects many physiological systems in the body and induces biochemical changes affecting pain perception and behavior [4, 52]. The relevance of the problem is dictated by the fact that stress-induced conditions/diseases (e.g. coronary heart disease, arterial hypertension, diabetes mellitus, peptic ulcer disease, Graves' disease, malignancies, depression, reproductive disorders) are socially significant, affecting the individual's emotional state, motivation, and behavioral functions. A number of mediators and neurotransmitter systems are involved in stress-induced analgesia - endocannabinoid, adrenergic, opioidergic, nitricoxidergic, and others [1, 53]. The ECS system interacts with various signaling pathways as well as other receptor families and affects a wide range of physiological processes. The ECS is thought to regulate neuronal activity through anatomical circuits involved in the stress response and through its ability to modulate neurotransmitter release [54 - 56]. A growing body of evidence suggests that stress modulates endogenous N/OFQergic signaling. N/OFQ-NOP receptor system modulates pain transmission, stress and anxiety, learning and memory, and food intake [23, 24]. Nociceptin has diverse effects in different species and pain models, depending on the site of injection and dosage, with hyperalgesic effects at the supraspinal level and antinociceptive effects in the periphery [57, 58]. N/OFQ is alleviate behavioral and sensory responses during

stress, such as fear responses [59] and analgesia [24].

Endogenous neuropeptides have been studied to discover potent analgesics, without the concomitant side effects of opioids. The incorporation of amino acids into the molecule of natural biological peptides leads to the production of analogues with biological activity, which is of scientific interest.

CONCLUSIONS

For the first time, original results were obtained for the relationships between N/OFQ analogues and the endocannabinoid neurotransmitter system after chronic stress (CIS). The results described confirmed the participation of the ECS system in the analgesic effects of nociceptin and novel analogues after stress (CIS). The analgesic effects of N/OFQ analogues are influenced by CB1-receptors agonist (AEA) and antagonist (AM251) after CIS. Our study demonstrated that substitution of Lys at positions 9 and 13 in the molecule of nociceptin decreased significantly the pain threshold of the newly synthesised analogue [Orn⁹,Orn¹³]N/OFQ(1-13)NH₂ after chronic stress.

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