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Final dissertation

“ Characterization of Neuropeptide S Derivatives at the Human NPS Receptor ”

Supervisor
Dr. Davide Malfacini

Candidate *Lal SARPKAYA*
Student ID number: 2086000

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List of abbreviations:

AM	acetoxymethyl
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
FIU	fluorescence intensity units
Fluo-4	fluorophore
GPCR	G-protein coupled receptor
HBSS	Hanks' Balanced Salt Solution
HEK	Human embryonic cells
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
IP_3	inositol trisphosphate
NPS	Neuropeptide S
NPSR	Neuropeptide S receptor
Phe	phenylalanine
PIP_2	phospholipid phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
s.e.m.	standard error of the mean

Abstract

Neuropeptide S (NPS) is the endogenous 20-amino acid neuropeptide that selectively binds and activates a previously orphan GPCR, referred to as the neuropeptide S receptor (NPSR). Activation of NPSR induces intracellular calcium mobilization and cyclic AMP (cAMP) accumulation, indicating coupling to Gq and Gs signaling pathways and resulting in increased cellular excitability. The NPS/NPSR system is an important signalling pathway in both central and peripheral nervous systems and plays a role in the modulation of multiple physiological functions, including anxiety-related behaviour, learning and memory processes, locomotion, food intake, sleep-wake regulation, pain and drug addiction. In this thesis, the activity of Neuropeptide S derivatives at the human NPS receptor was assessed. Receptor activation was evaluated using a fluorescence-based calcium mobilization assay. In the calcium mobilization assay, NPS acted as a full agonist, displaying a high maximal efficacy (E_{max}= 208%) and high potency (pEC_{50}= 8.88), in line with literature results. Consequently, NPS peptide derivatives (NPS(2-12)-NH_2, NPS(1-10)-NH_2, Ac[D-Cys^5]NPS(2-12)-NH_2 and Ac-[tbu-D-Gly^5]NPS(2-12)-NH_2) were evaluated alongside with tetrapeptide ligands SFKN-NH_2 and SFRN-NH_2 and a series of derivatives (Ac-Cha-KN-NH_2, Ac-Cha-RN-NH_2, Ac-SFKN-NH_2, Ac-SFRN-NH_2, S-Cha-KN-NH_2 and S-Cha-RN-NH_2). Results in terms of detailed in vitro pharmacological profiles are reported in this thesis work, and these findings may support further studies of innovative pharmacological entities acting at the NPS/NPSR system.

1. INTRODUCTION

1.1 G-protein Coupled Receptors

G-protein coupled receptors (GPCRs) are one of the largest and most diverse families of membrane receptors in the human body [7], particularly in the central nervous system (CNS). They mediate cellular responses to extracellular stimuli, including hormones, neurotransmitters, peptides and sensory signals. Due to their pharmacological profile, GPCRs regulate several physiological processes like neuronal excitability, endocrine regulation and synaptic transmission.

GPCRs transmit extracellular signals by coupling to heterotrimeric G proteins ($G\alpha$, $G\beta$, $G\gamma$) [7], which regulate intracellular effector enzymes and thereby control the production of second messengers. Depending on the G protein subtype, receptor activation can lead to distinct signaling outcomes. For example, activation of Gs stimulates adenylyl cyclase and increases intracellular cyclic adenosine monophosphate (cAMP) levels, whereas activation of Gq promotes phospholipase C signaling and induces intracellular Ca^{2+} release. These second messengers are widely used as indicators of GPCR activation in vitro, since changes in intracellular Ca^{2+} and cAMP concentrations directly correspond to receptor activity which can be quantitatively assessed in cell-based assays. The Neuropeptide S receptor (NPSR) was initially identified as an orphan GPCR, as its endogenous ligand had not yet been discovered. The discovery of neuropeptide S (NPS) as its physiological ligand established the NPS/NPSR system [1] and allowed the characterization of receptor signaling and the development of agonists and antagonists.

1.2 Neuropeptide S and NPS receptors

Neuropeptide S (NPS) is a 20-amino acid neuropeptide that functions as the endogenous ligand of NPSR [1, 5]. NPSR expression is observed in multiple brain regions, including areas associated with arousal, stress responses and wakefulness, as well as behaviors involved in feeding behavior and addiction. Activation of the NPS/NPSR system has been shown to modulate several physiological and behavioral processes [4, 5]. Administration of NPS increases wakefulness and locomotor activity, while reducing anxiety-like behavior. In addition, NPS has been implicated in learning and memory processes, regulation of food intake and modulation of drug-seeking behavior.

At the cellular level, NPSR activation induces both intracellular calcium mobilization and cAMP accumulation [2], demonstrating coupling to Gq- and Gs-dependent signaling pathways. This dual signaling profile results in enhanced neuronal excitability and represents an important feature of

NPSR pharmacology. Because receptor activation can be quantitatively assessed through calcium mobilization assays and cAMP accumulation assays, these systems are used to evaluate the potency and efficacy of NPS and its synthetic derivatives. Such pharmacological characterization is essential for distinguishing full from partial agonists.

1.3 Gq Signaling and Calcium Mobilization

Although NPSR is known to couple to both Gq and Gs pathways, for this particular research, we focus on the Gq-mediated signaling pathway of NPSR and calcium mobilization. After agonist binding, NPSR activates Gq proteins, leading to the stimulation of phospholipase C (PLC). Activated PLC hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2), generating inositol trisphosphate (IP_3). IP_3 then binds to its receptors on the endoplasmic reticulum and induces the release of Ca^{2+} into the cytosol. The increase in intracellular Ca^{2+} levels can be used to measure NPSR activation through Gq signaling (figure 1). This response was quantified using a fluorescence-based calcium mobilization assay.

The calcium mobilization assay is a cell-based fluorescent method that measures increases in intracellular calcium (Ca^{2+}) after ligand binding to a G protein-coupled receptor (GPCR). In this assay, cells expressing the human NPS receptor are loaded with a Ca^{2+}-sensitive fluorescent dye (Fluo-4). Upon receptor activation, intracellular calcium levels increase, resulting in a proportional increase in fluorescence intensity.

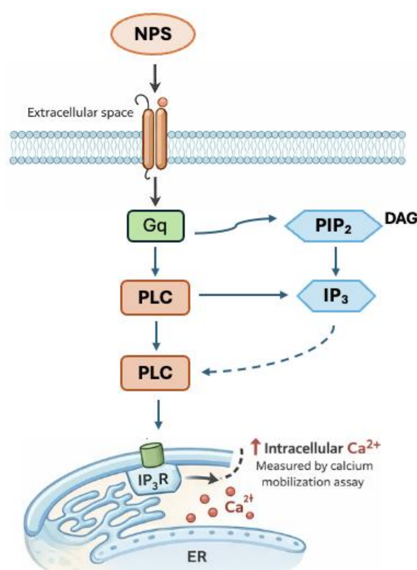

Figure 1: Gq-mediated signaling pathway of Neuropeptide S receptor (NPSR).

1.4 Aim of the Study

The aim of this study was to evaluate a series of NPS-derivatives at the human NPS receptor using a fluorescence-based calcium mobilization assay. By testing different concentrations of each compound, concentration response curves were generated to determine their potency (pEC_{50}) and maximal effect (Emax). These values were then compared to those of the reference agonist NPS to assess how structural modifications influenced Gq-mediated receptor activation.

2. MATERIALS AND METHODS

2.1 Cell Cultures: Human Embryonic Kidney Cells (HEK293 cells) expressing the human neuropeptide S receptor isoform NPSR-Ile107 were used for the experiments.

2.2 Solutions:

2.2.1 Growth Medium Buffer Solution with antibiotics: HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Hygromycin B (100 µg/mL), sodium pyruvate, and penicillin–streptomycin to maintain stable receptor expression (figure 2). Cells were grown in T75 culture flasks and maintained in this medium and incubated at 37°C, in an atmosphere containing %5 CO_2 (figure 3).

Dulbecco Modified Eagle Medium (DMEM) + HyG B (Hygromycin B) (100µg/mL) + sodium pyruvate (1mM) + penicillin streptomycin (100mg/mL)

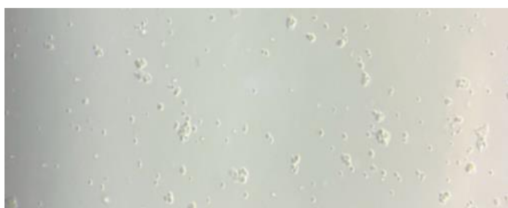

Figure 2: HEK293 cells before

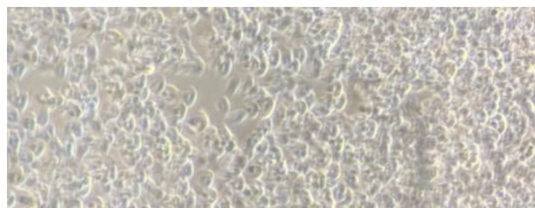

Figure 3: HEK293 cells after two days in culture.

2.2.2 HBSS Buffer Solution:

HBSS (500mL): KCl (5.4 mM), KH2PO4 (0.44 mM), NaCl (137mM), NaHCO3 (4.2 mM), Na2HPO4 x 7 H2O (0.25 mM), CaCl2 (1.3 mM), MgSO4 x 7 H2O (1mM), glucose (5 mM)

+ HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) (20 mM, pH 7,4 (20mM)

2.2.3 Ligand Buffer Solution: This solution was used to load the compound plate.

HBSS Buffer solution + BSA (Bovine Serum Albumin) (5% m/V)

2.2.6 Probenecid Solution:

probenecid (2.5 mM) + HBSS Buffer + NaOH (sodium hydroxide)

2.2.5 Fluorophore Solution (Loading solution):

HBSS Buffer + fluorophore (3 µM) + pluronic (0.02%) + probenecid solution (2,5 mM)

2.2.6 Brilliant Black Solution (Dye loading solution):

HBSS Buffer + probenecid solution (2.5 mM) + Brilliant Black (500µM)

2.3 List of Compounds used in Calcium Mobilization Assay: The places of the compounds on the ligand place were switched each assay to consider plate effect.

NPS

NPS(2-12)-NH_2

NPS(1-10)-NH_2

SFKN-NH_2

SFRN-NH_2

Ac-SFKN-NH_2

Ac-SFRN-NH_2

Ac-FKN-NH_2

Ac-FRN-NH_2

S-Cha-KN-NH_2

S-Cha-RN-NH_2

Ac-Cha-KN-NH_2

Ac-Cha-RN-NH_2

Ac-[tbu-D-Gly^5]NPS(2-12)-NH_2

Ac[D-Cys^5]NPS(2-12)-NH_2

c[NPS (2-12)]

2.4 Calcium Mobilization Assay with FlexStation 3 - In vitro studies:

Approximately 24 hours before performing the intracellular calcium mobilization assay, HEK293 cells expressing the human NPS receptor were seeded into a 384-well plate with 50,000 cells per well in 50 µL of growth medium. Cell numbers were counted using a CytoSMART automated cell counter to ensure an accurate number of cells before plating. To prepare the cells for counting, the

culture medium was removed from the culture flask, and the cells were washed with PBS. The cells were detached from the surface of the flask by using 2 mL of trypsin solution (0.5 g/L; EDTA 0.2 g/L) which was incubated at 37°C for 3-4 minutes. The proteolytic activity of trypsin was stopped by adding 8 mL of fresh culture medium. The cells were then aspirated from the flask and transferred into a sterile 15 mL Falcon tube. Finally, 10 µL of the cell suspension was loaded into the Burker cell counting chamber, so that the cells could be counted using the automated cell counter.

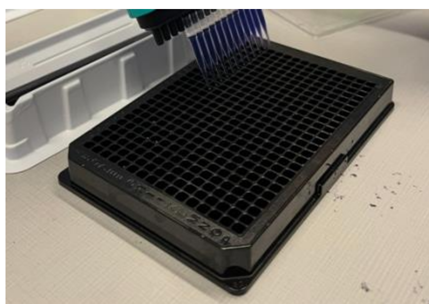

Figure 4: Cell plate with 384 wells (16 rows x 24 columns) during Brilliant Black solution is added.

After seeding, the cell plate was incubated for 24h at 37ºC in a humidified atmosphere containing 5% CO_2 to allow cells to attach to the plate surface and form a confluent monolayer. Following incubation, the culture medium was removed from the plate and cells were incubated with fluorophore solution of 30µL/well for 1 hour (figure 4).

The calcium mobilization assay is a cell-based fluorescent method that measures increases in intracellular calcium (Ca^{2+}) after ligand binding to a G protein-coupled receptor (GPCR). Fluo-4 is a membrane-permeable fluorescent calcium indicator that enters cells by passive diffusion. Once inside the cell, intracellular esterases cleave the acetoxymethyl (AM) groups, trapping the fluorescent dye within the cytosol. Probenecid is included in the loading solution to prevent leakage of the dye from the cells.

Since the dye is hydrophobic, the loading solution also contains pluronic which improves the solubility of Fluo-4 and facilitates its uptake into the cells. Any extracellular dye that does not enter the cells is masked by Brilliant Black dye, which reduces background fluorescence during fluorescence measurements.

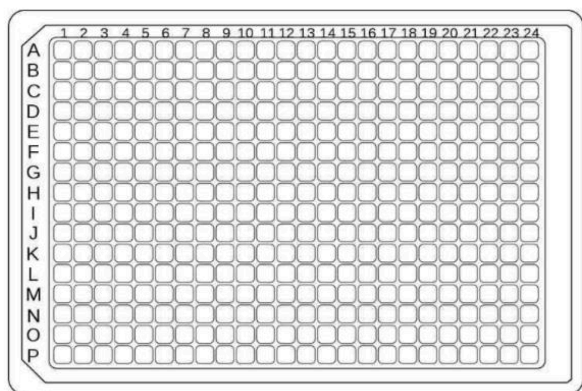

Figure 5: The compound plate.

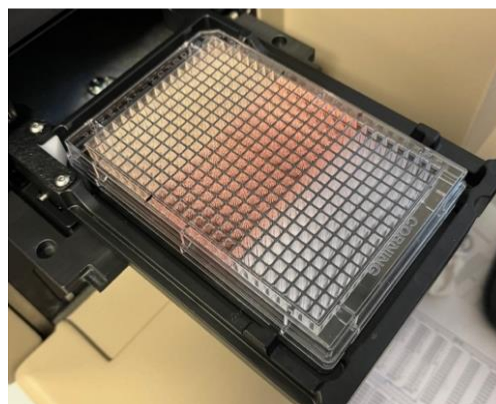

Figure 6: The compound plate in Flex Station III.

At the same time compound plate is prepared in a 384-well compound plate with a flat bottom (figure 5). The layout was organized to generate 1:10 serial dilutions of the compounds. Row A contained 100 µL of buffer only and served as a control without compound. In row B, 95 µL of buffer and 5 µL of the compound at the highest concentration were added. From rows C to I, 90 µL of buffer were added to each well and vertical serial dilutions (1:10) were performed by transferring the solution from the previous row. The procedure was repeated in the second half of the plate. In row I, 95 µL of buffer and 5 µL of compound were added to prepare the highest concentration for the second dilution series. From rows J to P, 90 µL of buffer were added and vertical serial dilutions (1:10) were again performed. Finally, row P contained 100 µL of buffer without compound, serving as an additional control (figure 6).

To minimize potential plate effects, the positions of the tested compounds were changed between experiments, ensuring that compounds were tested in different regions of the plate across independent assays.

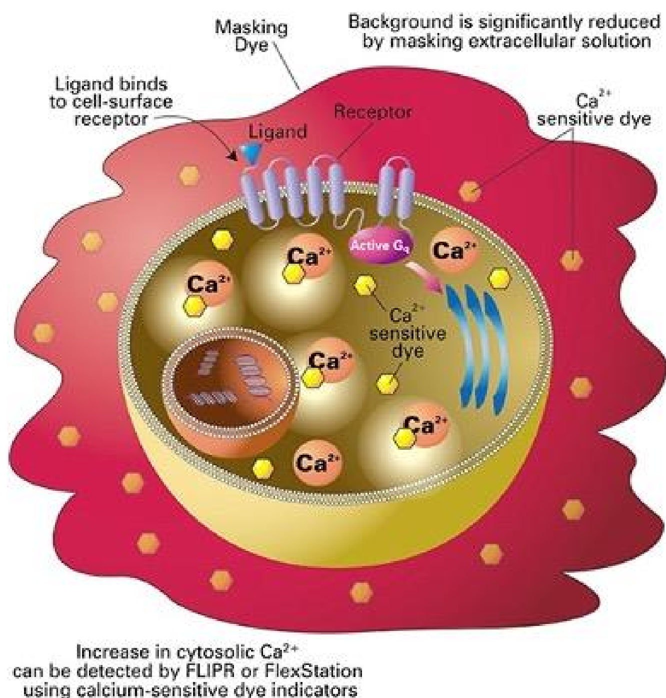

Figure 7: Schematic diagram of the intracellular calcium mobilization assay.

After this preparation, when the ligand binds to the cell-surface receptor, intracellular signaling pathways are activated, resulting in an increase in cytosolic Ca^{2+} concentration (figure 7). This increase is detected by the calcium-sensitive dye as a change in fluorescence intensity. The change is then measured using FlexStation III, which allows detection of increases in intracellular Ca^{2+} in response to stimulation.

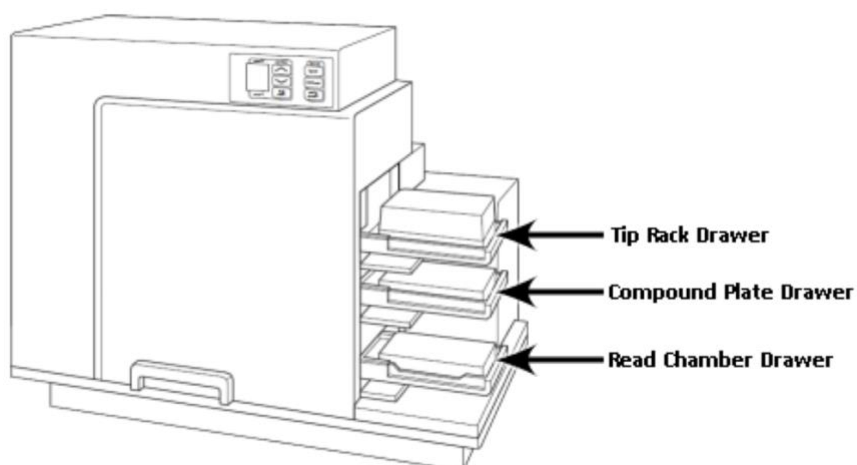

Figure 8: *FlexStation III used in the calcium mobilization assay*

FlexStation III is a multi-mode microplate reader which measures absorbance, fluorescence intensity, polarization, luminescence and time-resolved fluorescence (figure 8). It uses automated

pipetting with an 8-channel pipettor to dispense ligands in triplicate during the assay. The instrument is equipped with an automated pipetting system, and in this study an 8-channel pipettor was used to dispense ligands in triplicate during the assay. FlexStation III contains an advanced optical detection system based on dual monochromators and a high-power xenon-arc flash lamp [6]. The monochromators allow precise selection of excitation and emission wavelengths without the need for fixed optical filters.

For the calcium mobilization assay, cells were previously loaded with the calcium-sensitive fluorescent dye Fluo-4. The fluorophore was excited at λ 488 nm, and the emitted fluorescence was detected at λ 525 nm. Changes in fluorescence signal correspond to changes in intracellular Ca^{2+} concentration following receptor activation. The fluorescence signal recorded by the instrument is expressed as fluorescence intensity units (FIU).

2.5 Data Analysis

In this study, ligands were tested at multiple concentrations to construct concentration–response curves and evaluate their pharmacological profiles at the NPS receptor. From the concentration response curves, potency was determined by calculating the EC_{50} value, which represents the concentration of a ligand required to produce 50% of the maximal response. For easier comparison between compounds, potency was expressed as pEC_{50}, defined as the negative logarithm of the EC_{50} value.

Additionally, efficacy was determined based on the maximal response (Emax) produced by each ligand, using NPS as the reference agonist. These parameters allow classification of compounds as full agonists, partial agonists, or inactive at the NPS receptor.

Data analysis and curve fitting were performed using GraphPad Prism software. All data are expressed as means ± standard error of the mean (s.e.m.) of n experiments. Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline. Agonist potencies are given as pEC50 = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist.

Concentration response curve to agonists were fitted with the following equation:

Effect = baseline + (Emax-baseline)/(1+10^((LogEC50 - X)*HillSlope))

3. RESULTS AND DISCUSSION

In this thesis, the activity of Neuropeptide S (NPS) and its derivatives was assessed in vitro in HEK293 cells stably expressing the Ile107 isoform of the human NPS receptor (NPSR) using a fluorescence-based calcium assay. The activity of each compound was compared to the NPSR endogenous agonist NPS, which served as a reference agonist.

3.1 Pharmacological Characterization of NPS

In this assay, NPS was used as the reference agonist for comparison with the tested derivatives. NPS produced a concentration-dependent increase in intracellular Ca^{2+} levels, as shown by the fluorescence signal recorded in the calcium mobilization assay. The compound displayed a maximal response (E_{max}) of 208% and high potency, with a pEC_{50} value of 8.88, indicating strong receptor activation (figure 9).

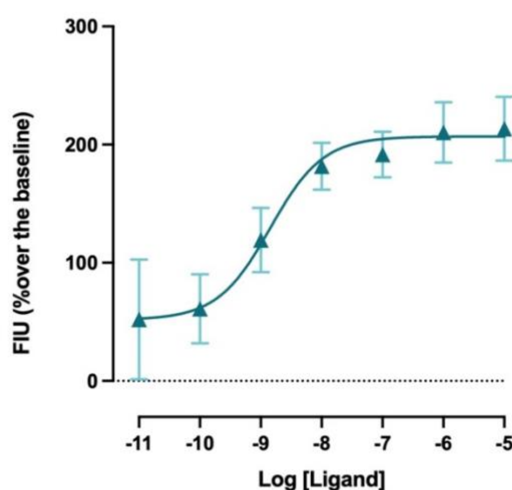

Figure 9: Concentration-response curve of NPS. Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of NPS. Data are presented as mean ± SEM of five experiments performed in triplicate.

3.2 Truncated Derivatives of NPS

The truncated peptides NPS(2-12)-NH_2 and NPS(1-10)-NH_2 mimicked the stimulatory effect of the natural occurring peptide NPS, maintaining high efficacy comparable to that of NPS; thus behaving as full agonists, showing maximal responses (respectively: E_{max} of 216% and 236%) and high potency with pEC_{50} values close to that of the reference. In contrast, the cyclic derivative c[NPS (2-12)] displayed high maximal efficacy but reduced potency with pEC_{50} value of 6.07 (figure 10).

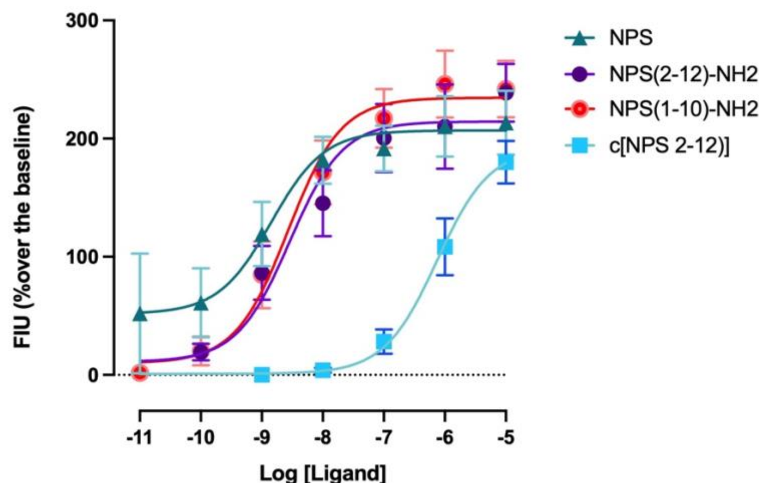

Figure 10: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of NPS(2-12)-NH2, NPS(1-10)-NH2 and c[NPS(2-12)]. Data are presented as mean ± SEM of five experiments performed in triplicate.

3.3 Tetrapeptide Ligands

Tetrapeptides SFKN-NH_2 and SFRN-NH_2 maximal responses were high (209% for SFKN-NH_2 and 184% for SFRN-NH_2), and comparable to that of NPS; therefore, behaving as full agonists in the present assay. Both compounds displayed lower potency compared with NPS. Their pEC_{50} values were 7.61 and 7.68, respectively (figure 11).

3.4 N-Terminal Acetylated Tetrapeptides

The tetrapeptides SFKN-NH_2 and SFRN-NH_2 act as references for the acetylated analogues. Ac-SFKN-NH_2 and Ac-SFRN-NH_2 showed weak stimulatory effects only in the micromolar range of concentrations (figure 11).

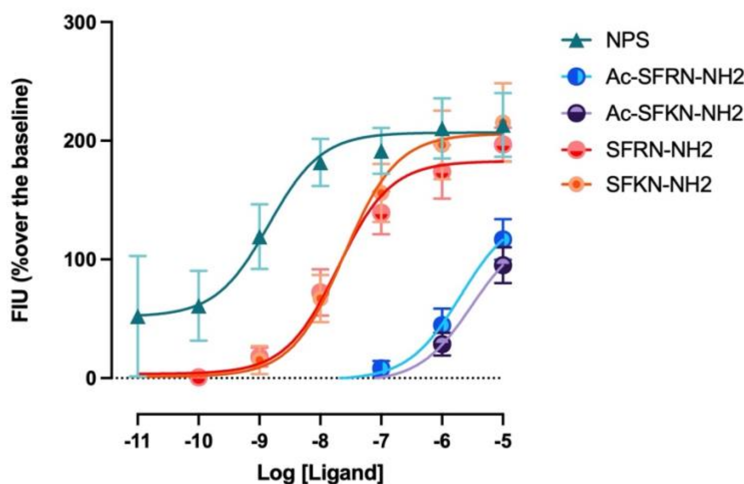

Figure 11: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of SFKN-NH_2, SFRN-NH_2, Ac-SFKN-NH_2 and Ac-SFRN-NH_2. Data are presented as mean ± SEM of five experiments performed in triplicate.

In contrast, Ac-FKN-NH_2 and Ac-FRN-NH_2 were capable of activating the receptor. Both compounds showed reduced potency compared with the reference ligand. The maximal responses were 195% and 163%, respectively, while the pEC_{50} values were 6.72 and 6.61 (figure 12).

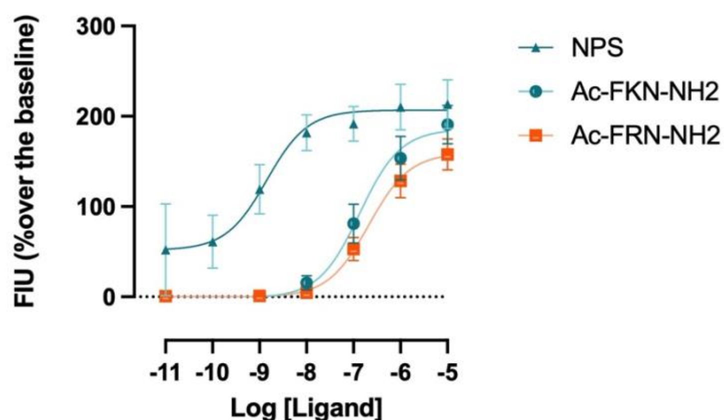

Figure 12: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of Ac-FKN-NH_2 and Ac-FRN-NH_2. Data are presented as mean ± SEM of five experiments performed in triplicate.

3.5 Cha-Substituted Derivatives

The substitution of Phe (F) with cyclohexylalanine (Cha) in the Ac-FKN-NH_2 and Ac-FRN-NH_2 scaffolds was not tolerated. In fact, Ac-Cha-KN-NH_2 and Ac-Cha-RN-NH_2 effects were weak only in the high micromolar range of concentrations tested (figure 13).

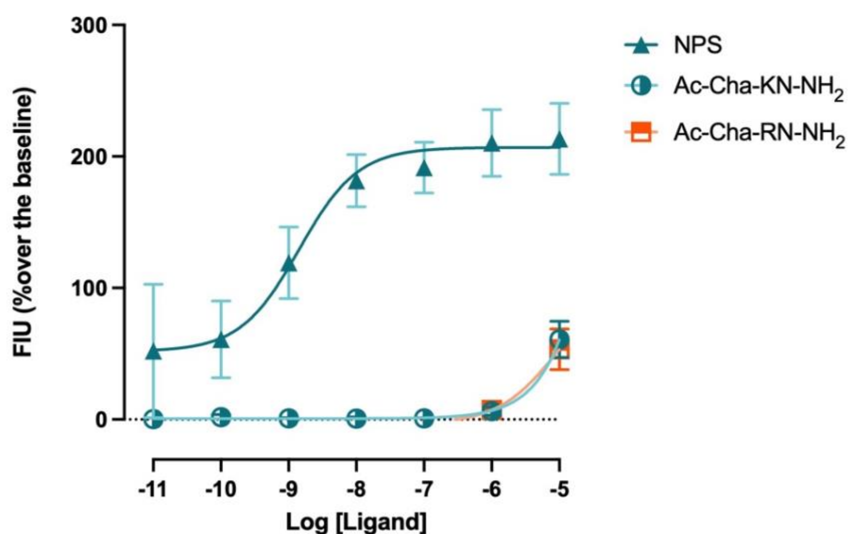

Figure 13: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of acetylated Cha derivatives. Data are presented as mean ± SEM of five experiments performed in triplicate.

Phe[2] in the parental tetrapeptides SFRN-NH_2 and SFKN-NH_2 were substituted with Cha. The occurring peptides S-Cha-KN-NH_2 and S-Cha-RN-NH_2 showed weak stimulatory effects only in the micromolar range of concentrations (figure 14).

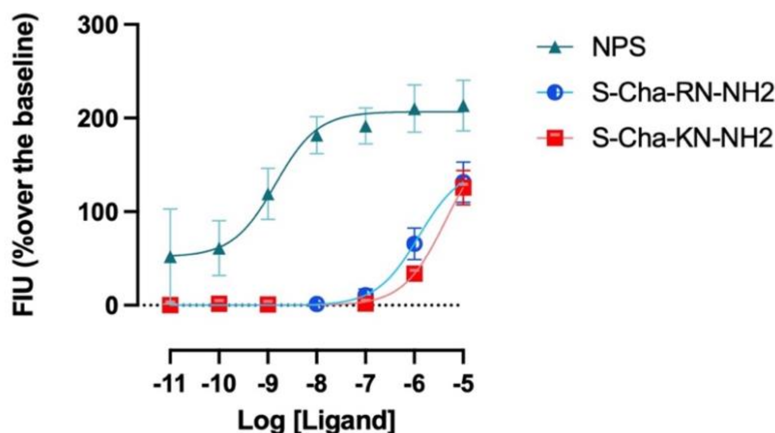

Figure 14: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of Cha-substituted tetrapeptide derivatives. Data are presented as mean ± SEM of five experiments performed in triplicate.

3.6 Position 5 Modified NPS Derivatives

Finally, a separate series of NPS derivatives was tested. Synthetic peptides contain modifications at position 5. The compound Ac-[tbu-D-Gly^5]NPS(2-12)-NH_2 was inactive as agonist (figure 15); therefore, future studies will be carried out to evaluate its potential as NPSR antagonist.

In contrast, Ac[D-Cys^5]NPS(2-12)-NH_2 showed diminished a maximal response (125%) compared to NPS, and reduced potency (pEC_{50} 7.91) (figure 15).

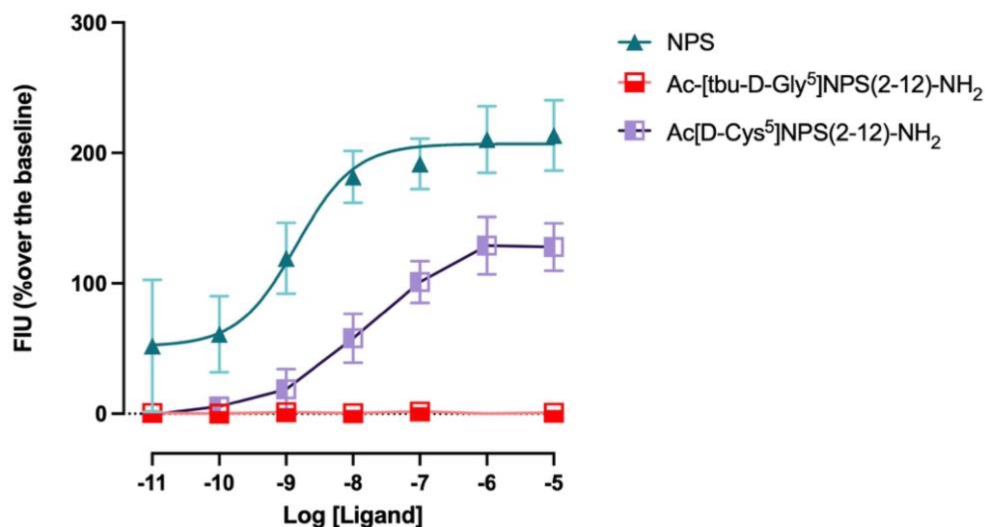

Figure 15: Intracellular calcium mobilization assay in HEK293T hNPSR-Ile107 cells. Concentration–response curve of position 5 modified NPS derivatives. Data are presented as mean ± SEM of five experiments performed in triplicate.

These derivatives are based on the truncated peptide NPS(2-12), which has previously been shown as the shorter fragments still retaining receptor activation [11], and they also have specific substitutions at position 5 – known as relevant for efficacy at the NPSR - together with N-terminal acetylation of the peptide sequence. The results obtained are in line with literature data and corroborate the notion that position 5 can strongly influence receptor activation [9].

A summary of the pharmacological parameters obtained for all tested compounds is presented in Table 1.

Table 1: Summary table of compounds and their pharmacological parameters.

LIGANDS	Emax (% ± SEM)	pEC50 (CI95%)
NPS	208 ± 22	8.88 (8.35 - 9.41)
NPS(2-12)-NH2	216 ± 28	8.56 (7.9 - 9.19)
NPS(1-10)-NH2	236 ± 25	8.59 (7.93 - 9.24)
SFKN-NH2	209 ± 32	7.61 (7.08 - 8.13)
SFRN-NH2	184 ± 17	7.68 (7.23 - 8.13)
Ac-SFKN-NH2	<6	
Ac-SFRN-NH2	<6	
Ac-FKN-NH2	195 ± 20	6.72 (6.05 – 7.40)
Ac-FRN-NH2	163 ± 17	6.61 (6.41 – 7.07)
S-Cha-KN-NH2	<6	
S-Cha-RN-NH2	<6	
Ac-Cha-KN-NH$_2$	<5	
Ac-Cha-RN-NH$_2$	<5	
Ac-[tbu-D-Gly^5]NPS(2-12)-NH$_2$	inactive	
Ac[D-Cys^5]NPS(2-12)-NH$_2$	125 ± 18	7.91 (7.19 – 8.63)
c[NPS (2-12)]	200 ± 18	6.07 (5.53 – 6.61)

4. CONCLUSION

This study investigated the pharmacological activity of several neuropeptide S derivatives at the human neuropeptide S receptor using a fluorescence-based calcium mobilization assay. The aim of the work was to evaluate how different NPS derivatives with structural modifications may influence receptor activation through the Gq-mediated signaling pathway.

The endogenous ligand NPS produced a strong concentration-dependent increase in intracellular Ca^{2+} levels and acted as a full agonist in the assay. The results for NPS were used as a reference for comparison with the other compounds. Evaluation of the truncated peptides NPS(2-12)-NH_2 and NPS(1-10)-NH_2 showed that shorter fragments of the NPS sequence can still retain receptor activity with reduced potency compared to the full peptide. This confirms that shortening of the peptide sequence may affect ligand-receptor interactions.

The tetrapeptides SFKN-NH_2 and SFRN-NH_2 activated the NPS receptor with lower potency compared to NPS, in line with literature findings [11]. Together with other chemical alterations, these results highlight that improving the pharmacological profile of the available SFKN-NH2 and SFRN-NH2 tetrapeptides is rather difficult. Further studies are needed to understand how these molecules harbor their interaction with NPSR binding pocket. Nevertheless, with these results we support the available knowledge by pointing out which moieties and overall ligand size must be preserved. This might support the discovery of new potential peptidomimetic drug candidates.

Modifications at position 5 of the NPS sequence also affected receptor activation. While the derivative Ac[D-Cys^5]NPS(2-12)-NH_2 showed partial agonist activity with reduced potency and maximal response, Ac[tBu-D-Gly^5]NPS(2-12)-NH_2 remained inactive within the tested concentration range. These observations support that substitutions at position 5 can significantly influence the pharmacological properties of NPS derivatives.

Overall, the results obtained in this research underline the importance of the peptide sequence and structural modifications. Small changes such as truncation, amino acid substitution, or N-terminal acetylation can strongly influence receptor activation and potency. These findings contribute to a better understanding of the structure–activity relationships of NPS derivatives and may support future research targeting the NPS/NPSR system.

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