Published in final edited form as:

Peptides. 2010 January ; 31(1): 130. doi:10.1016/j.peptides.2009.10.015.

Importance of extracellular loop one of the Neuropeptide S receptor for biogenesis and function

Stewart D. Clark^a, Ha T. Tran^a, Joanne Zeng^a, and Rainer K. Reinscheid^{a,b,c,*}

Stewart D. Clark: sdclark@uci.edu; Ha T. Tran: hat@uci.edu; Joanne Zeng: yzeng@uci.edu; Rainer K. Reinscheid: rreinsch@uci.edu

^a Pharmaceutical Sciences, University of California, Irvine, 147 Biological Sciences Administration, Irvine, CA 92697, USA

^b Department of Pharmacology, University of California, Irvine, 360 Med Surge II, Irvine, CA 92697, USA

^c Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, CA 92697, USA

Abstract

Neuropeptide S (NPS) is the endogenous ligand of a formerly orphan G protein-coupled receptor (GPCR). The NPS receptor (NPSR) belongs to the subfamily of peptide GPCRs and is widely expressed in the brain. NPS promotes arousal and induces anxiolytic-like effects after central administration in rodents. Previously, we have reported that the N107I polymorphism in the human NPS receptor results in a gain-of-function characterized by an increase in agonist potency without changing agonist binding affinity. We have extended our findings by investigating pharmacological and biochemical consequences of mutations in the vicinity of position 107. Alanine substitutions were made for D105 and N101, and stable clones were analyzed for agonist-induced changes of intracellular Ca^{2+} . Receptor protein expression was monitored by Western blot and flow cytometry. The mutation D105A produced receptors that have a ~200-fold higher EC_{50} despite elevated total receptor protein and surface expression compared to cell lines expressing the parental receptor NPSR-N107. The mutation N101A resulted in slightly reduced agonist potency without affecting the ability of the protein to form functional receptors. Stable NPSR-A101 clones show little expression of the fully glycosylated form. However, NPSR-A101 receptors are expressed on the cell surface and are functional, suggesting that full glycosylation is not required for receptor function. Our studies suggest that N-linked glycosylation is not important for receptor biogenesis or function, and that residue D105 might be critical for receptor binding.

Keywords

Neuropeptide S; GPCR; extracellular loop one; biogenesis; mutagenesis; pharmacological chaperone

* Corresponding author: Rainer K. Reinscheid, Phone: (949) 824-9228, Fax: (949) 824-4855, rreinsch@uci.edu, University of California, Department of Pharmaceutical Sciences, 310C Med Surge 2, Irvine, CA 92697.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Recently the orphan G protein-coupled receptor GPR154 was shown to be the receptor for a novel neuropeptide, termed Neuropeptide S (NPS). NPS was named after the N-terminal serine residue which is conserved across species [23]. NPS has been found in mice to induce behavioral arousal and was shown to produce anxiolytic-like effects in paradigms that measure fear or responses to novelty [15,23]. In humans, multiple single-nucleotide polymorphisms (SNPs) and several splice variants have been identified for the NPS receptor (NPSR) gene and its transcript. We have previously investigated a SNP that codes for a single amino acid change (N107I) in NPSR, and found that the I107 variant displays higher agonist efficacy with no change in binding affinity [17]. Furthermore, genetic linkage of this variant to “average bedtime” [6], and gender-specific association of NPSR N107I genotypes with panic disorder [13] have been reported recently. However, it is currently unclear if the altered pharmacology of the two NPSR variants is functionally linked to the genetic associations.

The N107I polymorphism is located in the first extracellular loop (ECL1) of the receptor. There has been accumulating evidence from other neuropeptide GPCRs that ECL1 plays a critical role in ligand binding and signal transduction. In ECL1 there are three defined regions which have been proposed to have distinct functions (Fig. 1). Two are well conserved motifs (WXFG, DXXCR) which have been linked to signal transduction [8,10]. The third region is located between transmembrane domain two (TM2) and the WXFG motif and has been reported to be involved in ligand binding [8,20]. This region displays low homology between receptor types (Fig. 1B), but is highly conserved across different species for a particular receptor (Fig. 1C). In general, polar amino acids of ECL1 were shown to be involved in ligand binding and signal transduction. In the region between TM2 and the WXFG motif of NPSR only a small number of polar amino acid residues are found. The most prominent is D105 which is a charged acidic residue. It is possible that this charged group and the polar side chain of N101 may interact with highly conserved basic or polar amino acid residues in NPS [16].

In this study, a mutational analysis of the ECL1 subregion of NPSR between the TM2 and WXFG motif was completed. Profound changes in biogenesis and ligand binding were found after alanine substitution of N101 and D105. We demonstrate that D105 is required for high affinity binding of NPS but not of the NPSR antagonist SHA 68 [12]. Taken together with our previous findings for the N107I polymorphism [17], our data point to an important role for polar amino acids within ECL1 for receptor conformation, signal transduction and binding.

2.0 Methods and Materials

2.1 Reagents

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): brefeldin A, cycloheximide, tunicamycin; which were directly added to the cell culture medium to the final concentrations and for the durations indicated under “Results.” Antibodies were obtained from the following sources: anti-HA HA.11 monoclonal antibody (16B12; Covance, Emeryville, CA), anti-p44/42 MAP kinase (Cell Signaling Technology, Danvers, MA), anti-Mouse IgG, and anti-Rabbit IgG (Abcam Inc., Cambridge, MA). Tissue culture reagents including Dulbecco's Modified Eagles medium (DMEM), fetal bovine serum (FBS), hygromycin, and Hank's balanced salt were obtained from Invitrogen (Carlsbad, CA). The NPSR antagonist SHA 68 was synthesized as described [12]. NPS was synthesized by the Peptide Proteomic Centre, Brain Research Centre, University of British Columbia (Vancouver, BC, Canada) and stock solutions were dissolved in water. PNGase F and all restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All other chemicals were obtained from Sigma-Aldrich.

2.2 Epitope tagging and mutagenesis

Complementary oligonucleotides containing coding sequences for a signal peptide and the hemagglutinin antigen (HA) tag (agcttatgaagacgatcatcgccctgagctacatcttctgcctgggtgttcgccgactatccttacgacgtgcctgattatgc and tcgagcataatcaggcagctcgtgaaggatagtcggcgaaaccaggcagaagatgtagctcagggcgatgatcgtcttc ata) were purchased from Sigma Genosys (The Woodlands, TX). Oligonucleotides were annealed and ligated into pcDNA3.1 containing NPSR I107 or NPSR N107 that had been cut with HindIII and XhoI. Single colonies were selected and plasmid DNA was extracted to verify the sequence. The NPSR N107 (GeneBank accession number: NM 207172) cDNA was used as template for all further mutations. Point-directed mutagenesis was performed by Retrogen, Inc. (San Diego, CA) and all constructs were sequence verified.

2.3 Stable expression of mutant receptors and functional characterization

HEK293 T cells were transfected with the human NPS receptor cDNA constructs cloned into pcDNA 3.1 using LipofectAmine (Invitrogen, Carlsbad, CA) and selected with 400 mg/l hygromycin for 14-21 days. Stable clones were plated in duplicate into black 96-well clear-bottom plates treated with poly-D-lysine and allowed to attach overnight. After loading with 1 μ M Fluo-4 AM indicator dye, cells were washed three times in assay buffer (Hank's balanced salt, 10 mM HEPES, 2.5 mM probenecid, pH 7.4). NPS (100 nM final concentration in assay buffer containing 0.1% bovine serum albumin) was added to each well and mobilization of intracellular Ca^{2+} was monitored over 180 seconds (FLIPR, Molecular Devices, Sunnyvale, CA). Peak fluorescence values were extracted and normalized to cell densities and then used to select positive responding clones. For constructs showing functional responses, 2-6 individual clones were selected and expanded further. Clones from non-responding constructs were pooled for further analysis of protein expression. Individual clones were plated again for dose-response analysis to various concentrations of NPS in triplicates (24 wells per clone total). EC₅₀ values were calculated with GraphPad Prism (GraphPad, San Diego, CA).

2.4 Western blotting

Western analysis of receptor expression was done as previously described [21]. Cells were washed two times with phosphate-buffered saline (PBS), harvested, and lysed using RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM PMSF). Cell lysate in Laemmli buffer was heated for 45 min at 37 °C and separated on a 7.5% Tris-glycine gel followed by transfer to polyvinylidene difluoride membrane (Pierce Biotechnology, Inc., Rockford, IL). Epitope-tagged receptors were detected using anti-HA (16B12) antibody in concert with a horseradish peroxidase-conjugated secondary antibody and the ECL Plus chemiluminescent reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Protein content was determined for all lysates by bicinchoninic acid assay (Thermo Scientific, Pittsburgh, PA) and equal amounts of protein were loaded to lanes that were to be compared to one another. For loading controls extracellular signal-regulated kinase (ERK) was detected using the p44/42 MAPK (Erk1/2) Antibody (#9102; Cell Signaling Technology, Danvers, MA).

2.5 Radioligand binding assay

HEK 293T cells stably expressing human NPSR were seeded into 24-well plates and cultured for 48 hr. [¹²⁵I]Tyr¹⁰-NPS (human) was purchased from NEN Perkin Elmer (Boston, MA). Nonspecific binding was determined in the presence of 1 μ M unlabeled human NPS. The binding assay was carried out as described [19]. In brief, cells were washed with PBS first and then incubated with radioligand with or without unlabeled NPS peptide in DMEM containing 0.1% bovine serum albumin at 20°C for 1.5 hr. Cells were washed five times with cold PBS

and lysed with 1 N NaOH. Bound radioactivity was counted in a MicroBeta liquid scintillation counter (EG&G Wallac, Gaithersburg, MD) and corrected for counting efficiency. Data from triplicate incubations were analyzed using GraphPad Prism.

2.6 PNGase F treatment

Cells were rinsed using ice cold PBS and then lysed for 1 h at room temperature with sodium phosphate lysis buffer (125 mM sodium phosphate buffer (pH 7.4), 10 mM EDTA, 1% SDS, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM PMSF). Samples were then run through a 21 1/2 gauge needle to reduce viscosity. Insoluble material was precipitated by centrifugation at $14\,000 \times g$ at room temperature. Two 75 µl aliquots were prepared, with the remainder set aside for total protein determination. To each of the 75 µl aliquots 1/10 volume of 10% triton X-100 was added, and one unit of PNGase F was added to one of the aliquots. Both aliquots were then incubated for 1 h at 37°C. Samples were separated by SDS-PAGE and subjected to Western analysis, as described above.

2.7 Flow cytometry

Cells were grown to confluency in 6-well plates and then mechanically dislodged from the surface. Cells were collected by centrifugation and resuspended in 0.5-1 ml PBS. To fix the cells, paraformaldehyde to a final concentration of 1-2% was added and cells were incubated for 10 min at 37°C. After chilling on ice for 1 min, cells were again captured by centrifugation and resuspended in PBS, 0.5% BSA. Cells at a density of 5×10^5 cells/ml were incubated with Alexa Fluor 488-conjugated anti-HA antiserum (HA-Tag (6E2) mouse mAb; Cell Signaling Technology, Danvers, MA) for 30-60 min and then washed twice with PBS. Flow cytometry was carried out on a FACSCalibur flow cytometry instrument (Becton-Dickinson, San Jose, CA) by sampling at least 1×10^4 cells. Settings for forward-scatter and side-scatter were selected appropriately to ensure only viable cells were counted.

3.0 Results

3.1 Functional Screening

Over 50% of the randomly picked HA-tagged N107 and I107 clones (after antibiotic selection) produced functional responses in the FLIPR assay. Two individual clones of each construct were selected for further testing. The ranges of EC_{50} values for the two individual clones of each variant are very close to our earlier published results (I107 (clones #4 and 5), $EC_{50} = 1.6 \pm 0.7$ and 1.7 ± 0.3 nM, respectively; N107 (clones #2 and 4), $EC_{50} = 25.0 \pm 3.5$ and 4.9 ± 1.2 nM, respectively; Fig. 2 A & B; [17]), suggesting that the addition of the HA-tag has little or no influence on agonist activation of the receptor. As noted before, EC_{50} values for agonist stimulation of NPSR-I107 are lower than for NPSR-N107 and calcium signals tend to be higher for NPSR-I107 [17].

All further mutations were made in the NPSR-N107 background. The modification N101A slightly reduces agonist potency (two clones selected for further study, clones #2 and 6; $EC_{50} = 12.0 \pm 1.9$ nM and 42.0 ± 7.1 nM, respectively; Fig. 2C). The high number of positive clones (37 out of 69 clones analyzed were responding to NPS) indicates that this mutation does not affect the ability of the protein to form functional receptors. Conversely, alanine substitution of D105 abolished any measurable response in the FLIPR at the screening concentration of NPS (100 nM) in all 37 individual clones that were tested. We therefore pooled all 37 clones for further analysis by FLIPR, Western Blot and flow cytometry. The pooled D105A clones were reanalyzed by FLIPR using a higher concentration range of NPS and directly compared to the parental NPSR-N107 construct (Fig. 2D). It was found that the EC_{50} for NPS in pooled cells stably expressing D105A was ~200 times higher than the parental NPSR-N107 clone ($EC_{50} = 583.2 \pm 106.7$ nM; Fig. 2D). Together, these findings suggest that

position N101 plays a minimal role in signal transduction and ligand binding, while removing the charged side chain of D105 may either inhibit ligand binding and/or signal transduction.

3.2 Post-translational Modifications

Western Blot analysis indicated that both NPSR-N107 and NPSR-I107 receptor proteins occur as two distinct protein species in transfected cells (Fig. 3A). The lower molecular weight (MW) species runs as a 44 kDa protein while the high MW form appears to be about 52 kDa in size. These MW are calculated from comparison of band migration to that of a regression of standards separated along with the samples, the theoretical MW of HA-tagged NPSR-N107 is 44 kDa. The difference between the two predominant protein bands most likely reflects different levels of glycosylation of NPSR, which contains only one consensus site for N-linked glycosylation near its aminotermminus (N4). Western analysis of NPSR-A101 shows very low amounts of the high MW form, which would suggest either low surface expression, or that glycosylation is not required for efficient trafficking of NPSR (Fig. 3C). Surprisingly, the pool of stable NPSR-A105 clones showed extremely elevated protein expression, especially of the high MW band of the receptor protein, as seen in Western blot analysis (Fig. 3C). The lower MW form (44 kDa) is present, but not visible in the presented figures because of the overwhelming expression of the 52 kDa form. The expression of NSPR-A105 is so high in fact, that we consistently had to reduce the amount of total protein loaded to one-fifth in order to allow for clear visualization of the bands in Western analysis. The difficulty in obtaining a clear picture when the proteins are in such disparate amounts is evident in the loading control blot, probed for total amounts of extracellular signal-regulated kinase (ERK) (Fig. 4A, lower panel).

From work with other GPCRs we know that the low MW bands represent less mature species of the receptor protein [21]. This was indeed found to be the case for NPSR-N107 and NPSR-A101. PNGaseF is an amidase that cleaves between the innermost N-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. PNGaseF treatment of NPSR-N107 and NSPR-A101 resulted in a single band of 39 kDa in Western analysis (Fig. 3C). This indicates that the 52 and 44 kDa species represent higher glycosylated forms of the 39 kDa receptor protein. The lack of effect of PNGaseF treatment on D105A may be due to lower total protein loading, although, we have loaded more protein and still observed no effect (data not shown). The partial shift of the 52 kDa band of NPSR-A105 (Fig. 3C) likely reflects incomplete deglycosylation and may be due to the aggregation of receptor protein resulting in the protection of the innermost N-acetylglucosamine and asparagine residue from PNGase F. However, the portion of the glycan that remains accessible may be subject to non-specific activity of PNGase F, thus, explaining the small shift in size that is observed.

To establish that the patterns of expression we observed for each receptor construct are not due to clonal artifacts produced by stable expression, we transiently transfected each construct into HEK 293T cells. These cells were either not treated or treated overnight with tunicamycin to block the addition of N-linked glycans (Fig. 3D). The molecular weights of the bands for each of the receptor constructs are comparable in size and amount to those seen after stable transfection. Furthermore, as expected, tunicamycin treatment mirrors the results obtained after PNGaseF treatment (compare Fig. 3C & Fig. 3D). These data indicate that our results obtained from individual stable clones are specific to the constructs and not clonal artifacts.

It appears that the majority of 52 kDa species in lysates from the stable NPSR-A105 cell line are protein aggregates. In transiently transfected cells, which are lysed 48 hours after transfection, the amount of aggregated protein appears to be less, presumably because less time to form aggregates was available. Therefore, we were able to load 50% of the normal amount

of protein from lysates of transiently transfected cells and were able to visualize all the molecular species in the presented exposure (Fig. 3D).

3.3 Surface Expression

Flow cytometry was carried out to determine the presence and quantity of receptor protein on the cell surface. The stable clone of NPSR-N107 displayed significant quantities of receptor protein on the cell surface (Fig. 3B top panel; median fluorescence (MF) = 9.14 arbitrary units (a.u.)) as compared to control cells that do not express the antigen (HEK 293T, non-transfected; MF = 4.18 a.u.). NPSR-I107 showed even higher expression levels (Fig. 3B bottom panel; MF = 52.80 a.u.) which is twelve times higher than in NPSR-N107 cells. The increased cell-surface expression of NPSR-I107 could explain – at least in part - why NPSR-I107 clones tend to show higher maximal agonist-induced Ca^{2+} -responses in the FLIPR assay. NPSR-A101 (MF = 4.91 a.u.) displayed similar cell surface expression levels as the parental NPSR-N107 cells (MF = 4.78 a.u.; compare top & middle panel of Fig. 4B; non-transfected control MF = 3.08 a.u.). This result was unexpected, as both selected NPSR-A101 clones showed low overall expression of the receptor protein and in particular very low expression of the highly glycosylated form of the receptor protein in Western analysis (Fig. 3C & 4A). However, since the maximal responses for agonist-induced Ca^{2+} -mobilization in FLIPR were similar in both NPSR-A101 and NPSR-N107, our data indicate that full glycosylation of the receptor may not be necessary for membrane insertion. Taken together the data suggest that N-glycosylation may not be required for receptor biogenesis, function or surface expression. Since the high MW band is indicative of fully glycosylated receptor protein that has undergone posttranslational maturation in the golgi apparatus, it was not surprising to find large amounts of NPSR-A105 receptor protein on the cell surface by flow cytometry. In fact, the NPSR-A105 (MF = 20.72 a.u.) clones express fifteen times more cell-surface receptor protein than the parental NPSR-N107 construct (bottom panel of Fig. 4B).

3.4 Radioligand Binding to D105A

The D105A mutation results in an increased expression of the mature form of the receptor (52 kDa; Western analysis) and a concomitant increase in surface expression (flow cytometry). When taken together with the ~200 fold higher EC_{50} , this suggests that alanine substitution of D105 in NPSR causes either a drastic reduction in affinity or a perturbation of signal transduction. Therefore, radioligand binding of NPSR-A105 and NPSR-N107 was directly compared. Competitive displacement binding yielded an IC_{50} value for NPSR-N107 in the low nanomolar range (8.7 ± 1.9 nM), while NPSR-A105 showed no apparent binding (Fig. 5A). It should be noted that IC_{50} values greater than 200 nM cannot be determined because the association between radioligand and receptor would be too weak to be observed under the conditions of our binding studies.

3.5 Pharmacological chaperone effect (flow cytometry and FLIPR)

It has been previously found that GPCR expression can be upregulated by specific pharmacological agents, independent of their effects on receptor signaling. This phenomenon has been termed pharmacological chaperone (PhCh) effect [1,21]. The PhCh effect has been previously described for both wildtype GPCRs [14] and for naturally occurring mutants that are associated with genetic disorders (e.g. a mutation in the V2 vasopressin receptor causing nephrogenic diabetes insipidus [11]). Upregulation is not dependent on signaling through agonist stimulation, as antagonists and agonists similarly upregulate the receptor. In addition, only compounds that are permeable to the plasma membrane produce this increase in expression [21]. To investigate whether the poor ligand binding of D105A is due to poor receptor biogenesis (e.g. receptor folding) we took advantage of this phenomenon.

Our experiments show that the NPSR antagonist SHA 68 [12] indeed can act as a PhCh on NPSR-N107. A time course of treatment reveals increase of the mature form of the receptor protein over time, peaking at 12 hours (Fig. 6A). This upregulation of the mature form of the receptor was also seen to be dose dependent (Fig. 6B). This is a pharmacologically specific event, as an unrelated GPCR (dopamine receptor subtype 4) treated with SHA 68 does not show upregulation of the mature glycosylated form (~60 kDa) of the receptor protein (Fig. 6C).

The increase in the high molecular form of the receptor due to overnight treatment with SHA 68 was also seen for NPSR-A101 and NPSR-A105 (Fig. 4A). To verify that the increases in the mature form of the receptor are indeed surface-expressed receptor protein, flow cytometry was performed. SHA 68 treatment produced an increase of surface-expressed receptor protein in each of the constructs, which was proportional to the increases in expression of the highly glycosylated forms of the receptors in Western analysis for NPSR-N107 (MF = 67.32 a.u.; fourteen times that of untreated) and NPSR-A101 (MF = 52.33 a.u.; ten times that of untreated), but not for NPSR-D105 (MF = 46.68 a.u.; two times that of untreated) (compare Fig. 4A & 4B).

The PhCh effect may be attributed to two potential mechanisms: these drugs either stabilize the mature receptor after it has left the ER or they stabilize a folding intermediate of the receptor within the ER. To discriminate between these two possibilities, we blocked NPSR export from the ER to the Golgi using brefeldin A. Brefeldin A treatment quickly results in disappearance of Golgi stacks and Golgi markers are found within the endoplasmic reticulum [3,4,7,24]. In addition, newly synthesized proteins become retained in the endoplasmic reticulum [9] as core glycosylated forms of the receptor (note the slightly larger molecular weight after brefeldin A treatment). Upon cotreatment with SHA 68 and brefeldin A there is a marked increase of NPSR expression (Fig. 6D). These observations indicate that SHA 68 probably exerts its effects by promoting the correct folding of NPSR within the ER, thus preventing degradation of a significant proportion of the newly synthesized receptor pool.

The PhCh approach also allows for investigating the question whether despite large amounts of surface-expressed receptor protein the low binding affinity observed in NPSR-A105 is due to aberrant protein folding. The use of a pharmacological chaperone may rescue this receptor and restore functionality. This technique has been used for a variety of GPCR mutants with perturbed biogenesis [1]. Therefore, we investigated the effect of overnight SHA 68 treatment in all NPSR constructs, followed by washout, and subsequent agonist activation. As shown in Fig. 4C, all NPSR constructs displayed an increase in maximal agonist response after SHA 68 treatment and drug washout. This is expected as the total number of surface-expressed receptors was increased (Fig. 4B). There was, however, no change in the EC₅₀ of NPSR-A105 after pharmacological chaperone treatment (data not shown), indicating that the protein might be correctly folded but unable to bind the agonist properly, as demonstrated in our radioligand binding experiments.

3.6 Schild analysis of antagonist binding to NPSR-A105

The PhCh effect of SHA 68 on NPSR-A105 biogenesis indicates that despite the poor affinity for NPS, this receptor mutant is still able to bind to the synthetic NPSR antagonist SHA 68. In order to determine the affinity of SHA 68 at NPSR-A105 we performed experiments to investigate whether SHA 68 can inhibit NPS-mediated calcium mobilization in the same dose range as it does at NPSR-N107. Technical limitations to assessing antagonist potencies are given by the relatively low affinity of NPSR-A105 to the agonist NPS, requiring very high agonist concentrations to achieve substantial receptor activation. Therefore, we estimated the affinity of the antagonist by interpolating equiactive agonist concentrations for calculation of dose ratios (DR). EC₅₀ values were not used as the E_{max} values could not be calculated due to

the poor agonist potency of NPS at NPSR-A105. Estimates of dose ratios from moderately low responses were used (5000, 7500, and 10000 fluorescent counts). From these dose ratios the K_b was calculated from the control curve and each dose-response curve obtained in the presence of SHA 68 ($K_b = [\text{antagonist}]/(\text{DR} - 1)$). Using this approach we obtained a K_b value of 118.6 ± 25.4 nM for SHA 68 at NPSR-A105 (Fig. 5B). This value is less than one order of magnitude different from what we previously reported for antagonism at NPSR-N (16.9 nM; [12]). These data indicate that although NPS has poor affinity for NPSR-A105, antagonist affinity seems less affected by the D105A mutation of NPSR.

4.0 Discussion

In the present study we have investigated the functional role of amino acids in the first extracellular loop (ECL1) of NPSR, located in close vicinity to the N107I polymorphic site that was previously found to significantly influence agonist potency [17]. We mutated ECL1 residues D105 and N101 to investigate the role of these amino acids in receptor function. The choice of these residues was made based on two criteria: they are polar and are outside ECL1 regions that are generally conserved among peptide GPCRs. We did not mutate the central part of ECL1 because it contains the sequence WRFTG which is reminiscent of the WXFG motif found in many rhodopsin-like GPCRs [10]. The W/YRFXG motif is also found in the V1a vasopressin receptor, which is most closely related to NPSR (~23% amino acid identity) (Fig. 1B), therefore this sequence is not unique to NPSR and thus not likely to play a role in ligand/receptor interaction. Increasing evidence suggests that mutations of the WXFG motif disrupt receptor activation but not ligand binding, where the motif operates downstream of ligand binding but upstream of the TM bundle switch mechanism [10]. The carboxyterminal part of ECL1 contains another motif highly conserved among peptidergic GPCRs; the canonical sequence DXXCR. This region is located at the interface between TM3 and ECL1, and is likely engaged in the formation of a disulphide bond between ECL1 and ECL2. This sequence motif has been shown in a number of other GPCRs to be involved in signal transduction [8,22]. The preservation of the WXFG and DXXCR motifs in GPCRs which have structurally diverse ligands suggests that these motifs are not involved directly in ligand binding. Therefore, we did not make mutations in these regions, but rather concentrated our efforts on the aminoterminal part of ECL1 proximal to the second transmembrane domain and in close vicinity to the polymorphic site N107I. This part of ECL1 displays the lowest level of sequence conservation across a number of peptide GPCRs, but is perfectly conserved among species orthologs of NPSR, and might thus be important for ligand selectivity and/or binding.

Profound changes in biogenesis and ligand binding were found after alanine substitution of N101 and D105. The N101A mutation produced a significant reduction in the expression of the fully glycosylated form of the receptor, albeit without decreasing cell surface expression. Despite the reduction in glycosylated receptor, cells expressing NPSR A101 displayed functional agonist responses similar to the parental NPSR-N107. These results suggest that neither residue N101 nor complete N-linked glycosylation are required for binding or signal transduction. Conversely, although mutation of D105 does not appear to affect surface expression of NPSR-A105, this amino acid might be critically involved in high affinity binding of NPS.

In many incidences, only a fully glycosylated GPCR is able to traffic effectively enough to insert into the outer membrane of the cell, and then be accessible to agonists [5]. Stable clones for NPSR-I107 consistently show higher levels of the more mature N-glycosylated 52 kDa form of the receptor protein than clones expressing NPSR-N107. This finding is consistent with previous results which used ELISA to measure surface expression in transfected cells [2]. The N107I polymorphic site is located between the WXFG motif and TM2, and therefore, both our present observations and the previous data indicate that amino acids of the first

extracellular loop are important for receptor trafficking and/or biogenesis. From our data using SHA 68 as a pharmacological chaperone, it appears that some of the difference in surface expression could be due to attenuated biogenesis of NPSR-N107 as compared to a more efficient expression of NPSR-I107. Due to the fact that the expression of both NPSR-N107 and NPSR-I107 are driven by a cytomegalovirus-derived (CMV) promoter, and that both constructs are lacking NPSR mRNA-derived untranslated regions, the increase in SHA 68-mediated protein expression is likely occurring at the protein level rather than from an increase in transcripts available for translation. Furthermore, the time course for the increase of protein expression is too short (increases appear after only two hours; Fig. 6A) for a significant contribution from newly transcribed mRNAs. In addition, the data obtained with brefeldin A treatment support the view that the increase in protein expression occurs due to the rescue of *de novo* synthesized protein from entering the endoplasmic reticulum-associated degradation (ERAD) pathway.

The two selected clones of NPSR-A101 mostly express incompletely glycosylated forms of the receptor protein, although these proteins seem to be expressed on the cell surface and are fully functional. Treatment with a pharmacological chaperone agent produces high levels of N-glycosylated forms of the receptor, which points to a receptor conformational cause for the incomplete glycosylation of NPSR-A101. Constitutive activity could also result in lower amounts of glycosylated receptor by the continuous endocytotic removal of the receptor from the cell surface. However, the maximal agonist responses in the two stable clones expressing NPSR-A101 as measured by the FLIPR assay are comparable to those observed in NPSR-N107 clones, indicating that constitutive activity is unlikely. In addition, there was no difference in cell surface expression as determined by flow cytometry. Therefore, our data suggest that residue N101 of NPSR is not important for signal transduction or receptor binding, and that N-linked glycosylation of the receptor may not be important for these attributes. However, the N101A mutation produces an altered conformation of the receptor protein that affects biogenesis.

Despite the enormous increase in expression of the high molecular weight form in cells expressing NPSR A105, it does not result in a correspondingly large increase in cell surface expression. This observation indicates that the mutation might facilitate protein biogenesis or inhibits identification by the protein quality control apparatus. Furthermore, the high molecular weight species of NPSR-A105 seem resistant to both tunicamycin and PNGaseF treatment. This apparent resistance could be due to an increase in post-translational modifications other than N-linked glycosylation. However, if the D105A mutation produces changes in the type of glycosylation, a change in size of the final receptor protein should also be expected. We found that the high molecular form of NPSR-A105 has exactly the same size as those of both NPSR-A101 and NPSR-N107, therefore, it seems unlikely that NPSR-A105 is being modified post-translationally in a substantially different way. Since tunicamycin blocks the addition of N-linked glycans, our data suggest that NPSR-A105 expressing cells contain a pool of receptor protein which is persistent and already glycosylated. Therefore, we speculate that the D105A mutation facilitates expression to such a degree that much of the protein is being aggregated or is being captured in alternative intracellular pools or a combination of these.

According to our functional assays, NPSR-A105 is still activated by NPS, albeit at ~200 fold higher agonist concentrations than the parental NPSR-N107. The drastic reduction in agonist binding affinity of NPSR-A105 could be caused by significant conformational changes of ECL1 or regions that directly interact with this part of the receptor, thus preventing agonist binding. However, it is more likely that the negatively charged side chain of D105 is directly involved in agonist binding by forming an ionic interaction with one of the positively charged residues in NPS (human NPS may carry up to five positive charges) that have been highly conserved during evolution [16]. In addition, the fact that a non-peptidic competitive antagonist

binds to NPSR-A105 (both Schild analysis and biogenesis experiments show this), suggests that the receptor protein is in its native conformation.

In an attempt to narrow the range of amino acids of NPS that potentially interact with D105 of NPSR-N107, receptor activation mediated by truncated forms of NPS was assayed. We have previously shown that human NPS 1-20 and 1-18 have similar EC_{50} values at NPSR-N107 (8.43 ± 1.47 nM and 10.9 ± 1.2 nM, respectively; [17]). These peptides, when compared to one another, displayed similar potencies in activating NPSR-A105 ($EC_{50} \approx 1000$ nM; unpublished data). The C-terminally truncated rat NPS 1-10 has an EC_{50} of 2090 ± 238 nM at NPSR-N107 [17] and no apparent activity at NPSR-A105 (unpublished data). A further truncated form, NPS 1-6, is able to activate NPSR-N107 ($EC_{50} = 1.31 \pm 0.32$ μ M [17]) and similar results have been reported by others [5,18], however this peptide is inactive at NPSR-A105 (tested up to a concentration of 10 μ M; unpublished data). Previous studies have also demonstrated the importance of amino acid residues 2, 3 and 4 of NPS for agonist activity while the C-terminal half of the peptide can tolerate almost any substitution [5,18]. Based on these observations it is reasonable to assume that the side chain of D105 in NPSR might interact with a positively charged residue within the amino terminal half of the peptide. Our preliminary investigation suggests that, while the residual binding capability of NPSR-A105 for NPS 1-20 may reside in its affinity for residues 11-18 of NPS, amino acids 1-10 are crucial for activation. Due to its positive charge, Arg³ is a likely candidate to interact with the D105 side chain. However, complementary mutational switch experiments are necessary to establish whether D105 directly interacts with Arg³ in NPS.

Taken together, the present data suggest that residue D105 in NPSR is required for high affinity NPS binding. However, D105 is not required for binding of the synthetic small molecule NPSR antagonist SHA 68. We also described data implying that complete N-glycosylation of NPSR is not required for receptor trafficking to the cell surface.

Acknowledgments

We thank Dr. Frederick Ehlert (UCI) for his contribution to our understanding of Schild analysis. We are also grateful to Drs. Chi Sum and Kathleen van Craenenbroeck for their constructive comments during the preparation of the manuscript. SDC is supported by a Canadian Institutes of Health Research Post-doctoral Fellowship. Work in the laboratory of RKR is supported in part by a grant from the National Institute of Mental Health (MH-71313).

References

1. Bernier V, Bichet DG, Bouvier M. Pharmacological chaperone action on G-protein-coupled receptors. *Curr Opin Pharmacol* 2004;4:528–533. [PubMed: 15351360]
2. Bernier V, Stocco R, Bogusky MJ, Joyce JG, Parachoniak C, Grenier K, et al. Structure-function relationships in the neuropeptide S receptor: molecular consequences of the asthma-associated mutation N107I. *J Biol Chem* 2006;281:24704–24712. [PubMed: 16790440]
3. Chardin P, McCormick F, Brefeldin A: the advantage of being uncompetitive. *Cell* 1999;97:153–155. [PubMed: 10219235]
4. D'Souza-Schorey C, Li G, Colombo MI, Stahl PD. A regulatory role for ARF6 in receptor-mediated endocytosis. *Science* 1995;267:1175–1178. [PubMed: 7855600]
5. Duvernay MT, Filipeanu CM, Wu G. The regulatory mechanisms of export trafficking of G protein-coupled receptors. *Cell Signal* 2005;17:1457–1465. [PubMed: 16014327]
6. Gottlieb DJ, O'Connor GT, Wilk JB. Genome-wide association of sleep and circadian phenotypes. *BMC Med Genet* 2007;8:S9. [PubMed: 17903308]
7. Gu F, Gruenberg J. ARF1 regulates pH-dependent COP functions in the early endocytic pathway. *J Biol Chem* 2000;275:8154–8160. [PubMed: 10713138]

8. Hawtin SR, Simms J, Conner M, Lawson Z, Parslow RA, Trim J, et al. Charged extracellular residues, conserved throughout a G-protein-coupled receptor family, are required for ligand binding, receptor activation, and cell-surface expression. *J Biol Chem* 2006;281:38478–38488. [PubMed: 16990262]
9. Klausner RD, Donaldson JG, Lippincott-Schwartz J, Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol* 1992;116:1071–1080. [PubMed: 1740466]
10. Klco JM, Nikiforovich GV, Baranski TJ. Genetic analysis of the first and third extracellular loops of the C5a receptor reveals an essential WXFG motif in the first loop. *J Biol Chem* 2006;281:12010–12019. [PubMed: 16505476]
11. Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, et al. Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Invest* 2000;105:887–895. [PubMed: 10749568]
12. Okamura N, Habay SA, Zeng J, Chamberlin AR, Reinscheid RK. Synthesis and pharmacological in vitro and in vivo profile of 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68), a selective antagonist of the neuropeptide S receptor. *J Pharmacol Exp Ther* 2008;325:893–901. [PubMed: 18337476]
13. Okamura N, Hashimoto K, Iyo M, Shimizu E, Dempfle A, Friedel S, et al. Gender-specific association of a functional coding polymorphism in the Neuropeptide S receptor gene with panic disorder but not with schizophrenia or attention-deficit/hyperactivity disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2007;31:1444–1448. [PubMed: 17669576]
14. Petaja-Repo UE, Hogue M, Bhalla S, Laperriere A, Morello JP, Bouvier M. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. *Embo J* 2002;21:1628–1637. [PubMed: 11927547]
15. Reinscheid RK. Neuropeptide S: anatomy, pharmacology, genetics and physiological functions. *Results Probl Cell Differ* 2008;46:145–158. [PubMed: 18204825]
16. Reinscheid RK. Phylogenetic appearance of neuropeptide S precursor proteins in tetrapods. *Peptides* 2007;28:830–837. [PubMed: 17293003]
17. Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, et al. Pharmacological characterization of human and murine neuropeptide s receptor variants. *J Pharmacol Exp Ther* 2005;315:1338–1345. [PubMed: 16144971]
18. Roth AL, Marzola E, Rizzi A, Arduin M, Trapella C, Corti C, et al. Structure-activity studies on neuropeptide S: identification of the amino acid residues crucial for receptor activation. *J Biol Chem* 2006;281:20809–20816. [PubMed: 16720571]
19. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;92 1 page following 696.
20. Silvente-Poirot S, Escriveau C, Wank SA. Role of the extracellular domains of the cholecystokinin receptor in agonist binding. *Mol Pharmacol* 1998;54:364–371. [PubMed: 9687578]
21. Van Craenenbroeck K, Clark SD, Cox MJ, Oak JN, Liu F, Van Tol HH. Folding efficiency is rate-limiting in dopamine D4 receptor biogenesis. *J Biol Chem* 2005;280:19350–19357. [PubMed: 15755724]
22. Wheatley M, Simms J, Hawtin SR, Wesley VJ, Wootten D, Conner M, et al. Extracellular loops and ligand binding to a subfamily of Family A G-protein-coupled receptors. *Biochem Soc Trans* 2007;35:717–720. [PubMed: 17635132]
23. Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, et al. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. *Neuron* 2004;43:487–497. [PubMed: 15312648]
24. Zeghouf M, Guibert B, Zeeh JC, Cherfils J. Arf, Sec7 and Brefeldin A: a model towards the therapeutic inhibition of guanine nucleotide-exchange factors. *Biochem Soc Trans* 2005;33:1265–1268. [PubMed: 16246094]

Figure One. Primary sequence and alignment of NPSR extracellular loop one

(A) Extracellular loop one (ECL1) of the NPS receptor contains the conserved motifs WXFG and DXXCR. The two amino acid residues mutated in this study are depicted by white circles (position 101 and 105). The natural SNP N107I is also depicted by a white circle. (B) Sequence alignment of human NPS receptor proximal ECL1 (between TM2 and the WXFG motif) with other human GPCRs, showing low similarity of this region between different receptor types. (C) High evolutionary conservation of the proximal ECL1 sequence in NPSR sequences from various mammalian species.

Figure Two. Functional responses of HA-tagged and mutant forms of NPSR

HA-tagged variants of NPSR were stably expressed in HEK 293T cells and assayed in FLIPR for NPS-mediated calcium mobilization: (A) NPSR-I107, (B) NPSR-N107, (C) NPSR-A101, and (D) NPSR-A105. Both the NPSR-A101 and NPSR-A105 construct are in the NPSR-N107 background. EC₅₀ values are calculated by non-linear regression, with each dose-response curve done in triplicate.

Figure Three. Expression analysis of HA-tagged and mutant forms of NPSR

(A) Western analysis of HA-tagged NPSR-N107 and NPSR-I107, showing expression of two distinct protein species at 44 and 52 kDa. (B) Flow cytometry shows that NPSR-N107 (line in top panel) has significant receptor protein (MF = 9.14 a.u.) on the cell surface as compared to control cells that do not express the antigen (MF = 4.18 a.u.) (HEK 293T, non-transfected; filled histogram on the left side of the figure). NPSR-I107 showed even higher expression levels (MF = 52.80 a.u.) (bottom panel). (C) When treated with PNGase F to remove N-glycans, both the NPSR-N107 and NPSR-A101 are seen to contract into a single 39 kDa band (Western analysis using anti-HA antibody). There is only a small shift in band size after PNGaseF treatment of NPSR-A105. For panels A-C representative clones are shown. (D) HEK 293T cells transiently transfected with constructs for NPSR-N107, NPSR-A101, and NPSR-A105 were treated overnight with tunicamycin (2 μ g/ml) to block the addition of N-glycans. (median fluorescence = MF; arbitrary units = a.u.)

Figure Four. Pharmacological chaperone effect of the NPSR antagonist SHA 68

(A) Overnight treatment with 1 μ M SHA 68 increased the amount of the high MW form of NPSR-N107, NPSR-A105, and NPSR-A101. Western analysis of NPSR was done using anti-HA antibody, while anti-ERK was used for loading controls. Due to the disparity of the amount of protein loaded for particular cell lines, two different exposures were taken in order to properly visualize the loading controls. (B) The same treatment increased the amount of surface expressed receptor (filled histogram: non-transfected cells (MF = 3.08 a.u.); black line: DMSO treated; grey line: SHA 68 treated; numbers shown in the figure are MF numbers for the respective cell line and condition). (median fluorescence = MF; arbitrary units = a.u.) (C) After washout of SHA 68, FLIPR analysis reveals a corresponding increase in maximal response

(NPS 10 μ M; * $p < 0.01$, ** $p < 0.001$ after t-test, response after SHA 68 treatment versus DMSO treated; shown as percent of response of vehicle treated).

Figure Five. Agonist and antagonist interaction with NPSR-A105

(A) Competition binding was performed using [125 I]Tyr 10 -NPS; NPSR-N107 $IC_{50} = 8.7 \pm 1.9$ nM, NPSR-A105 showed no apparent binding. (B) FLIPR analysis of NPSR-A105 was performed in the presence of increasing concentrations of SHA 68. The antagonist was able to inhibit NPS-mediated calcium mobilization (binding affinity for SHA 68 was calculated, $K_B = 118.6 \pm 25.4$ nM). n.t., no treatment

Figure Six. SHA 68 acts as a pharmacological chaperone

(A) Cells stably transfected with NPSR-N107 were treated with SHA 68 at a concentration of 1 μ M for varying times. (B) Cells were treated for 12 hours with different concentrations of SHA 68. (C) HEK 293T cells were transfected with either HA-tagged NPSR-N107 or HA-tagged dopamine receptor type 4 (HA-DRD4.2) and treated with 1 μ M SHA 68 or DMSO (0.1 % v/v final concentration) or not treated. (D) Treatment with brefeldin A (5 μ g/ml for 4 hrs) blocks the maturation of the high MW form of NPSR-N107. Further co-treatment with SHA 68 increases the amount of the immature form of NPSR-N107. For A-D, Western analysis of NPSR was performed using anti-HA antibody, while anti-ERK was used for loading controls.