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Didier Pelaprat

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Didier PELAPRAT[a, b*]

[a] U773 INSERM, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, BP416, 75870 Paris Cedex 18, France.

[b] EA 3512, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, BP416, 75870 Paris Cedex 18, France.

** Corresponding author:*

Dr Didier PELAPRAT

U773 INSERM,

Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard,

BP416,

75870 Paris Cedex 18, France.

Tel: (33)-1-44-85-63-10; fax: +33-1-44-85-63-06; Email address: pelaprat@bichat.inserm.fr

Abstract:

Three neurotensin (NT) receptors have been cloned to date, two of which, NTS1 and NTS2, belonging to the family of seven transmembrane domain receptors coupled to G proteins (GPCRs). NTS1 and NTS2 may activate multiple signal transduction pathways, involving several G proteins. However, whereas NT acts as an agonist towards all NTS1-mediated pathways, this peptide exerts agonist or antagonist activities depending on the considered NTS2-mediated pathway. Studies on these receptors reinforce the concept of independence between multiple signals potentially mediated through a single GPCR, generating a wide diversity of functional responses depending on the host cell and the ligand.

Keywords: Neurotensin, receptors, G protein

1. Introduction:

Neurotensin (NT), a 13-aminoacid peptide [13], acts as a neuromodulator in the brain and a paracrine or circulating hormone in periphery [38,74]. NT agonists or antagonists have been suggested to be of potential use for the treatment of pain, eating disorders, psychotic troubles, drug abuse and stress [6,36,64, 37]. Moreover, NT acts as a growth factor on different classes of normal and cancer cells, and NT-related ligands are proposed to represent valuable tools for tumor targeting [29,61].

Results from binding and pharmacological experiments have suggested the existence of different subtypes of NT receptors. For instance, two classes of NT binding sites were evidenced in rodent tissues, differing in affinity for NT and ability to bind the antihistamine drug, levocabastine [39,67]. The "high affinity site" (0.1-0.3 nM) was sensitive to Na^+ and GTP, did not bind levocabastine and showed a peak of expression around birth in some brain structures. The "low affinity site" (1-5 nM) was less sensitive to Na^+ and insensitive to GTP, bound levocabastine with high affinity (in rodent but not in human) and was more evenly distributed in brain structures, where its expression increased gradually until adulthood. Furthermore, NT analogs presented different relative potencies to elicit various pharmacological effects, such as modulation of dopaminergic transmission, hypothermia or analgesia, and these effects were differentially affected by antagonists [37,41].

The diversity of NT receptors has been confirmed through cloning experiments. Three NT receptors, termed NTS1, NTS2 and NTS3 (also referred to as NTR1, NTR2 or NTR3) have been cloned to date. NTS1 and NTS2 belong to the family of receptors with seven transmembrane spanning domains and coupled to G proteins (GPCRs), whereas NTS3 belongs to the family of sorting receptors [37,74].

Similarly to the previously reported "high affinity NT binding site", the rat and human NTS1s (424 and 418 amino acids, respectively) did not bind levocabastine and presented Na^+-

and GTP-sensitive NT binding sites [72,75]. Mutagenesis and molecular modeling studies revealed partial overlap between binding sites for NT and for the antagonist SR 48692 on rat NTS1 [5,37,40,57]. Residues Met208 in the fourth transmembrane domain (TM4) and Phe331 and Arg327 in TM6 interacted with both ligands. The SR 48692 binding site was located in an hydrophobic pocket involving other residues in TM6 and TM7, while the NT binding site resided closer to the extracellular side and encompassed additional interactions with residues in the third extracellular domain. Sodium sensitivity of agonist binding was born by Asp113 in TM1 [45].

Rodent [15,49] and human [76] NTS2s (416 and 410 amino acids, respectively) share only around 40% amino acid identities with the corresponding NTS1s, have shorter N-terminal extracellular tails and longer third intracellular loops. NTS2 corresponds to the previously described "low affinity NT binding site". Rodent NTS2s bind the histamine H1 antagonist levocabastine with high affinity, whereas human NTS2 presents lower affinity for this compound [15,49,76]. Affinity of NT for NTS2 is poorly sensitive to Na^+ and insensitive to GTP. Na^+-insensitivity was attributed to replacement of the Asp113 residue of rat NTS1 by Ala or Gly residues in corresponding positions of rat and human NTS2s, respectively [45].

By contrast with both NTS1 and NTS2, NTS3 belongs to the family of proteins characterized by a single transmembrane domain, a cysteine-rich domain and a short cytoplasmic C-terminal tail [50]. This 100-kDa protein was previously identified as sortilin, a protein involved in molecule sorting between the cell surface and intracellular compartments [48,58].

Investigations on the respective role of these receptors in mediating the effects of NT were greatly helped by the development of nonpeptide NTergic ligands such as SR 48692 [24]. This compound, first identified as a NTS1 antagonist, presented a higher affinity for this receptor than for NTS2, while the analog SR 142948A did not discriminate between these two

receptors [25]. Experiments performed with these compounds [25,37,71] as well as intracerebral injection of anti-receptor antisense oligonucleotides [19] or evaluation of knock-out mice [59], suggested that most of the NT effects could be attributed to stimulation of NTS1. However, the functional importance of NTS2 and NTS3 might still be largely underestimated. Several arguments suggest that NTS2 mediates analgesic effects of NT [19,44,81]. Recent data otherwise revealed a role of NTS3 in mediating NT effects on microglial migration, cytokine/chemokine expression and proliferation of cancer cell lines [17,18,47]. NTS3/sortilin was also found to be a co-receptor of p75NTR, mediating pro-NGF-induced apoptosis [54].

Interactions between NT receptors and G proteins were suggested through pioneering works showing that NT induced inositol phosphate (InsP) production [1,22,70], inhibited cAMP formation [10], and that the "high affinity NT binding site" was sensitive to guanine nucleotides [12]. Molecular studies thereafter indicated that most of these effects were attributable to NTS1. Since a majority of studies were performed on that receptor, this topic will represent a large part of this review. However, NTS2 also interacts with G-proteins, at least in some systems, and its striking differences with NTS1 at both molecular and functional levels, among which the finding that NT may behave as a NTS2 antagonist, raise fascinating questions. Furthermore, although not directly interacting with G proteins, NTS3 may modulate some of the G protein-dependent responses associated with NTS1 [46]. Moreover, NTS3 mRNA was detected in CHO cells [21]. Therefore, a possible involvement of this receptor in several effects solely attributed up to now to the other NT receptors has always to be kept in mind.

Although this review focuses on interactions between NT receptors and heterotrimeric G proteins, stimulation of these receptors may also lead to the activation of small G proteins, such as Rho-GTPases. Additionally, like other GPCRs, NT receptors probably interact with

many other partner proteins and may mediate some G protein-independent signaling events. These points will be briefly mentioned.

Finally, we will also see that, beyond mechanisms underlying NT function, studies on interactions between NT receptors and G proteins may also enlighten us on several issues of general interest in the field of GPCR modes of action.

2 Interactions between NTS1 and G proteins

2.1 One pluripotent receptor, several cells: how to combine economy with diversity

Common features of NT signaling in all studied natural or transfected systems expressing NTS1 were activation of phospholipase C, production of inositol phosphate and mobilization of intracellular calcium, suggesting that this receptor was a $G_{q/11}$-preferring receptor [37]. An involvement of these G proteins in the functional correlates of NTS1 receptor stimulation was also suggested by experiments showing that intracellular injection of an antibody against the common C-terminus of $G\alpha_q$ and $G\alpha_{11}$ inhibited the NT-induced alterations in function of ion channels [77]. However, NT was also found to induce inhibition of adenylyl cyclase and stimulation of arachidonic acid production through interaction with $G_{i/o}$-type G proteins in selected systems, such as CHO cells transfected with rat or human NTS1 [20,53,56] and rat neuroblastoma N1E115 cell line [11]. Furthermore, this peptide stimulated adenylyl cyclase through interaction with G_s in cells transfected with rat or human NTS1 [62,69,79] and in human pancreatic cancer cells endogenously expressing the receptor [31], but not in human colonic adenocarcinoma HT29 cells [1].

These data clearly indicated that, if NTS1 stimulation could lead to the activation of several G proteins, the expression of this potential depended on the host cell. Whether this phenomenon reflects differential accessibility to other cellular partners or differential compartimentalization of the receptor with G proteins remains to be established. Cell-

dependence was also observed for more distal intracellular events related to NTS1 stimulation. For instance, the $G_{q/11}$-related activation of the phospholipase C pathway could either be followed by an increase in cellular 3', 5'-cyclic guanosine monophosphate (cGMP) through activation of soluble guanylyl cyclase [2,70], or trigger a G_s-independent increase in cAMP levels through activation of calcium-dependent adenylyl cyclase [14]. Moreover, if NT stimulated both phospholipase C and protein kinase C in many cells, this peptide only marginally activated the latter branch of this pathway in HT29 cells [12]. Finally, activation of mitogen-activated protein kinases (MAPKs) upon NTS1 stimulation in transfected cells or some tumor cell lines involved either pertussis-sensitive or insensitive G proteins, or both [60].

The intracellular events triggered by NT or NT analogs were antagonized by SR 48692 [37,56]. In that sense, SR 48692 could be considered as an antagonist of all G protein-mediated consequences of NTS1 stimulation studied so far. One noticeable exception, however, was the Ca^{2+}-activated Cl^- current generated by NT in *Xenopus* oocytes expressing NTS1. Although this effect was generally recognized to be a consequence of the $G_{q/11}$-dependent phospholipase C activation, it was not antagonized by SR 48692 [9]. No explanation is yet available for this puzzling finding.

Some data also suggest that SR 48692 might behave as a NTS1 inverse agonist in certain systems. We observed that SR 48692 partially suppressed the constitutive InsP formation obtained upon expression of high amounts of NTS1 in sf9 insect cells [7]. Moreover, SR 48692 readily reversed the constitutive InsP production observed following mutation of the Phe358 residue present in the NTS1 seventh transmembrane domain [4].

2.2 One receptor, several domains, several G proteins, several ligands: independence and combinatorial pharmacology

An interesting finding was that the interactions between NTS1 and the diverse G proteins involved different receptor domains. Mutations in the NTS1 third intracellular loop suppressed agonist-induced InsP production without affecting cAMP accumulation, suggesting that this loop was essential for $G_{q/11}$ but not G_s activation [80]. Conversely, deletion of the receptor C-terminal domain suppressed activation of G_s- but not of $G_{q/11}$-related signal transduction pathways [28,69]. The receptor C-terminal domain was also shown to mediate the activation of $G_{i/o}$-related pathways, such as arachidonic acid release [53]. Although only based on activation of transduction pathways and not on a direct assessment of the physical interaction between the receptor and the corresponding G proteins, these data suggest that interaction with $G_{q/11}$ involves the NTS1 third intracellular loop, while interactions with $G_{i/o}$ and G_s employ the receptor C-terminal portion. Moreover, the fact that deletion or mutation of one intracellular domain completely suppresses some transduction pathways while leaving the others unaltered suggests a strikingly high degree of independence between functions mediated by individual domains, in spite of their belonging to the same protein. This characteristic can be further illustrated by data showing that mutation of the Phe358 residue in TM7 of rat NTS1 selectively confers a constitutive activity towards InsP but not towards cAMP production [4].

This possibility of independent activation of the various pathways is of fundamental importance for potential design of drugs with targeted pathway selectivity [26,32,33]. Indeed, there is no *a priori* reason that interaction between one receptor and different agonists should always result in the same set of domain conformations, leading to parallel degrees of activation of the corresponding G proteins. Separation of parameters at the level of the receptor molecule itself gives therefore the best potential to find agonist-selective patterns of response. This point may be illustrated by our recent demonstration that one NT(8-13) analog, EISAI-1, discriminated among pathways involving different NTS1 intracellular domains [69].

When compared with other agonists (NT, neuromedin N and JMV449), this compound presented strikingly low relative potency and efficacy to trigger the third intracellular loop-mediated $G_{q/11}$ activation, while being as efficient as other agonists towards the receptor C-terminal-mediated activation of G_s and $G_{i/o}$.(Fig.1) This phenomenon was observed in both transfected CHO cells overexpressing NTS1 and rat cortical neurons endogenously expressing this receptor, indicating that it did not merely reflect an artifactual situation generated by the expression system. The discriminative property of EISAI-1 could be attributed to the esterification of its C-terminus, preventing its interaction with Arg^{327}, a residue in TM6 which was found to play a major role in positioning the neighbouring third intracellular loop for an efficacious coupling to $G_{q/11}$ [5]. Disruption of this interaction led to an agonist-receptor complex that could still efficiently activate G_s and $G_{i/o}$, suggesting that it retained an adequate conformation of the more distal receptor C-terminus. This result was consistent with the existence of several agonist-selective receptor active states, differing in the conformation of one or several intracellular domains [32-35,69].

2.3 One receptor, one G protein: two distinct ways of influencing each other

Separation of parameters was also evidenced at the level of the bidirectional allosteric process that characterizes the receptor-G protein interaction. In this process, the presence of one partner or its additional interaction with a ligand may modify the conformation of specific domains of the other protein, including the ligand binding site itself. This process may translate into observable changes in the affinity of both kinds of ligands (receptor agonists and GTP), which are generally thought to be interdependent. However, data obtained on NTS1 give a clear illustration that this is not a general rule.

The ability of a G protein to increase the affinity of receptor agonists represents one of the two directions of the interaction process. This phenomenon is usually evidenced by the converse decrease in agonist affinity observed when the receptor-G protein interaction is

released, which can be achieved in the presence of guanine nucleotides (the "GTP shift"). The affinity of radiolabeled NT for wild-type NTS1 was decreased three-to fourfold in the presence of the stable GTP analog, GppNHp. Both NT affinity and GppNHp effect were strongly reduced after treatment of cell membranes with pertussis toxin, suggesting that the main G protein enhancing NT affinity was $G_{i/o}$ [20]. Furthermore, binding of radiolabeled NT to C-terminal truncated NTS1 expressed in CHO cells was totally insensitive to GppNHp, although this mutant receptor was still functionally coupled to $G_{q/11}$ activation [28]. More precisely, the C-terminal truncated receptor kept a high affinity for NT in the presence of GppNHp. This observation suggested that, in wild-type NTS1, the C-terminal part constrained the receptor in a lower affinity state, which was released by interaction with $G_{i/o}$. In the C-terminal truncated receptor, this constraint was already suppressed, and the presence of $G_{q/11}$ did not further increase NT affinity. Therefore, by contrast with $G_{i/o}$, interaction with $G_{q/11}$ did not appear to greatly alter the affinity of NTS1 agonists. Moreover, these data confirm that insensitivity of agonist binding to GTP analogs does not preclude an ability of the agonist-receptor complexes to trigger G protein activation [28, 33].

The ability of receptor agonists to increase the binding of GTP to the G proteins, indicative of the G protein activation, represents the reverse direction of the allosteric process. It may be evaluated by measuring the binding of [^{35}S]-GTPγS to membrane preparations. NT increased the binding of [^{35}S]-GTPγS in transfected CHO cells expressing rat NTS1 [20,27,53]. However, although the receptor was coupled to $G_{q/11}$, $G_{i/o}$ and G_s in this system, the agonist-induced increase in [^{35}S]-GTPγS binding was inhibited by pertussis toxin, suggesting that this parameter mainly reflected nucleotide exchange at $G_{i/o}$ [20,53]. This result was confirmed by experiments with fusion proteins consisting of various Gα subunits covalently linked to rat NTS1 C-terminus [23].

Therefore, in the case of NTS1, the existence of functional receptor-G protein interactions was not systematically associated with observable alterations in ligand binding to one of the partners. In particular, it appears that sensitivity of receptor agonist binding to GTP analogs and "functional coupling of the receptor to a G protein" should be regarded as independent parameters. Moreover, from a practical point of view, although most of the attention was paid to interactions between NTS1 and $G_{q/11}$, it is interesting to notice that the two kinds of measurements performed in order to follow the consequences of the receptor-G protein interactions were poorly sensitive to the presence of $G_{q/11}$, but rather reflected interaction between the receptor and $G_{i/o}$.

2.4 Integrating data into theoretical models: how one can be both useless and highly helpful.

These data may be discussed in terms of current models of ligand-receptor-G protein interactions, such as the cubic allosteric ternary complex (Fig. 2) [33]. In this model, like in the preceding extended ternary complex model [65], "inactive" receptor states are generically referred to as "R", and "active" receptor states are termed "R*". Ligands have the ability to alter the R*/R ratio, depending on the relative affinity for each receptor state. By contrast with the extended ternary complex model [65], the cubic model additionally assumes that both R and R* may interact with G proteins. However, by definition, only R* will be able to activate the G protein. Thus, this model separates the physical interaction from the activation process, which are associated events in the term “coupled to a G protein”.

In addition to the affinity constants involved in each bimolecular equilibrium, the model involves several parameters that characterize the influence of a given molecular species on the other partners (Fig.2A). Some parameters are related to the receptor ligand, such as α (ability of L to alter the R*/R ratio). Some others, such as β (ability of G to alter the R*/R ratio) or γ (ability of G to alter the affinity of the ligand for R), are related to the G protein. However, the values of these parameters are not intrinsic to the molecular species and are

only valid for interaction with these particular partners. In order to better discriminate between the abilities of R* to bind and to activate each G protein, this kind of description should be developed taking in account the transition from G to G* with additional parameters, such as μ, representing facilitation of this transition by the active receptor state (Fig.2B). Such a development will be particularly important if we want to introduce the possibility that a single R*might present different efficacies to activate distinct G proteins.

Within the limits set by the working capacity of the effector system, the maximal effect of a given agonist (E_{max}) in this system will be proportional to the amount of R*G* at saturation of the receptor forms to which it can bind. This amount is governed by the efficiency of the agonist and the G protein to induce the R-to-R* transition (α and β, respectively), the affinities of R and R* for the G protein (K_G and γ), and the efficacy of R* to activate the latter (μ). The potency (EC50) will reflect a combination of the agonist affinities for R, R*, and for their complexes with the G protein. If the effector working capacity reaches maximum before saturation ("receptor reserve"), this will produce a leftward shift of the EC50 value upon increased receptor expression [33]

Although such models theoretically provide sets of equations for analysis of experimental data, they remain of limited use since several parameter values are difficult to obtain experimentally. Moreover, these models do not take in account many important parameters, such as oligomerization of the receptor and interaction with other receptors or cellular proteins, that could greatly alter the functional responses. Nevertheless, examining their properties theoretically and experimentally as best we can may not only help to evaluate their accuracy to integrate experimental data but also raise several questions deserving future studies.

A series of comments concerns the effect of G proteins on receptor agonist affinity, reflected by the "GTP shift". In accordance with the observation that this phenomenon is

independent of G protein activation, these two events are described by different parameters in the cubic model. The ability of G to alter the affinity of the ligand for R is represented by γ, while the ability of a ligand to induce G protein activation will be conditioned by parameters such as α and μ. Since these values depend on the ligand, the receptor and the G protein, it can be expected that i) the intensity of the GTP shift might differ among agonists, ii) different values might be obtained on mutated receptors. Whereas the first point was not yet evidenced, greater "GTP shifts" for NT binding were observed following mutations of amino-acids in the third extracellular receptor loop [63]. Once again, no correlation could be found between this phenomenon and the degree of G protein activation by NT [63].

An interesting property of γ may also be pointed out. As illustrated in Fig. 2A, this parameter also reflects the ability of the ligand to alter the affinity of the receptor for the G protein. If only $G_{i/o}$ increases the affinity of NT for NTS1, it can thus be expected that NT facilitates formation of the NTS1-$G_{i/o}$ complex, but not that of NTS1-$G_{q/11}$ or NTS1-G_s. This would also mean that NTS1 already interacts tightly with both $G_{q/11}$ and G_s in the absence of ligand. Evaluating the validity of these hypotheses would provide a good support for the power of such models to describe the properties of these interactions.

A second point concerns the differential ability of agonists to activate the various signal transduction pathways. This phenomenon, previously termed "agonist-induced trafficking of receptor stimulus" [32-35], implied a high degree of independence between the activation of the different G proteins [26]. A first possibility to represent the interaction between a receptor and two G proteins is to generate a bicubic equation system by adding equilibria between R, R* and the second G protein (Fig. 3A). Differential activation of these G proteins by one agonist will be described by different values of the parameters traducing the ability of the active complex LR* to bind and transconform each G protein. Differences in the spectra of G protein activation induced by various agonists will be described through

stabilization of different R*s, endowed with different values of these parameters. A second proposition was the three-state model [42], in which R was in equilibrium with two active states R* and R**, each being able to bind and transconform only one of the G proteins (Fig. 3B). The ability of a given agonist to differentially activate the two G proteins, as well as differences between agonists, will only rely on their relative affinities for each receptor state. By contrast with the first approach, this second mode of description does not involve any multiplicity of agonist-selective receptor states, since the same R* and R** are considered whatever the agonist.

These two formalisms indeed underlie different assumptions which may not apply to all experimental situations. For instance, if the two G proteins interact with different domains of the receptor, as observed for $G_{q/11}$ and G_s, a single receptor molecule could possibly bind to two G proteins (Fig. 1A) and not only to one, as is assumes in the three-state model. Moreover, even if we hypothesized that each "active state" in the three-state model represented one domain, they could not be linked through successive equilibria since each domain is different from the other at a molecular level. Bipotent active receptor states, as represented in the bicubic model, seem therefore more accurate to describe such systems. From data presented in Fig.1 [69], it can thus be proposed that NT and some other agonists stabilize active states collectively referred to as R*1, presenting efficient conformations of both the receptor third intracellular loop and C-terminal portion for activation of all G proteins, while EISAI-1 stabilizes another class of active states, R*2, endowed with a less efficient conformation of the third intracellular loop towards activation of $G_{q/11}$. It should be noticed that such an hypothesis of multiple agonist-selective active states merely represents another expression of the plasticity of the macromolecule already postulated when we describe the allosteric transition R/R*.

Further examining the properties of the bicubic model brings one to question the assumption that successive equilibria link the two receptor-G protein complexes. For instance, will the formation of one complex necessarily be achieved at the expense of the other as suggested in Figs 3A and 3B? There are at least two scenarios in which this condition would be satisfied. The first situation is if one receptor molecule can simultaneously interact with the two G proteins. In that case, equilibria with each G protein would join through formation of a trimolecular complex, and competition between the two pathways would no longer occur. The second situation is if, conversely, we have to consider that the two G proteins interact with different non-interconverting receptor forms. These forms might differ in their post-translational modifications, or represent isolated receptor conformations stabilized by different microenvironments in discrete membrane compartments.

Indeed, recent data suggest that different forms of NTS1 may mediate InsP and cAMP production [63]. Although this receptor is often referred to as the "high affinity NT receptor", binding experiments performed with the radiolabeled antagonist [3H] SR48692 evidenced both the classical high affinity NT site (0.1 nM) and a second class of lower affinity NT sites (19 nM). Four mutations in the third extracellular loop resulted in the same kind of alterations: an increased proportion of low affinity over high affinity binding sites for NT, a 30-to 200-fold increase in EC50 for the stimulation of InsP production by NT and a 800- to 2500-fold increase for cAMP production. As pointed out by the authors [63], the detection of two classes of NT affinity suggested the presence of receptor forms which did not equilibrate under the experimental conditions. Furthermore, no correlation could be found between the relative affinity of NT or number of high affinity NT binding sites within this group of receptor mutants and the potencies or maximal effects of NT for activation of either InsP or cAMP production. For instance, the relative orders of EC50s and Emax values obtained for InsP production correlated with the low affinity site, while the characteristics of cAMP

production suggested an even lower affinity, yet undetected, NT site. Interestingly, the hypothesis of a low NT affinity site mediating InsP production applies not only to these mutants, but also to the previously reported mutation at the Asp113 position [45]. However, the influence of this mutation on cAMP production was not investigated in the latter study.

These data on mutated receptors suggest that activations of $G_{q/11}$ and G_s may have to be considered as involving non-interconverting receptor forms. This would therefore lead to a paradoxical description in which, despite each active state of NTS1 being theoretically bipotent, the two interactions would have to be formally represented through separate cubes (Fig. 4A).

Such a separate representation of the various pathways does not only apply to mutated receptors but would also be necessary to integrate another phenomenon involving the effects of EISAI-1 on the wild-type NTS1 [69]. In this study, we observed that a series of agonists displayed an inverse order of potencies for stimulating InsP formation as they did for enhancing cAMP production. These different pharmacological spectra were due to different relative potencies of EISAI-1 in the two assays when compared to other agonists. Indeed, as previously noticed [42], no model in which the different G protein-receptor complexes are linked by successive equilibria, like in the bicubic or the three-state model, may account for altered potency orders between agonists. Whatever the model, and even if the same R and R* could be taken for the two pathways, an "isolated pathway" mode [42] should be applied, describing the effect of a given agonist on each pathway through separate sets of equations (Fig. 4). It remains now to identify the characteristics of the system (compartmentalization of the G proteins, sequestration of various receptor populations in discrete plasma membrane microdomains, heterogeneity of post-translational modifications) which lead to such an apparent lack of equilibrium between the different pathways, and to better understand their functional implications at the cellular level.

It thus appears that, despite their huge oversimplification, theoretical models taking only in account the ligand-receptor-G protein complexes may represent useful tools to generate new hypotheses, even on mechanisms occurring under physiological conditions. However, such models are clearly not sufficient to integrate all experimental data. In this respect, a two-step model, involving the successive formation of metastable NT-NTS1 complexes and highly stable ligand-receptor adducts, was previously proposed in order to describe the role of Na^+ in modulating the NT-NTS1-G protein interactions in the cell environment [52]. Testing the validity of such new hypotheses would probably help us to refine our knowledge on the mechanisms underlying agonist-mediated NTS1 activation under physiological conditions.

2.5 Conclusion.

These considerations illustrate the variety of responses which may arise from the interactions between NTS1 and different G proteins, depending on the ligand and the host cell. However, this functional diversity may still be underestimated. In this respect, it was recently suggested that stimulation of this receptor by NT could also activate members of the pertussis-insensitive $G_{12/13}$ family, triggering the sequential activation of Rho-GTPases and NFkB-dependent pathways [82].

Finally, the functional responses associated with NTS1 can be greatly modified by other parameters, such as NTS1 oligomerization or interactions with other receptors and cell proteins. For instance, it was recently demonstrated that interaction between NTS1 and NTS3 decreased the potency of NT to produce InsP and also altered MAPK activation [46]. Most of the partners of NTS1, which could alter either its interaction with the G proteins or the functional consequences of these interactions, are still unknown. Although a large amount of data are now available on NTS1, many points remain to be elucidated.

3 Interactions between NTS2 and G proteins

Due to its characteristics (low sensitivity of NT binding to Na^+, insensitivity to guanine nucleotides), the "low affinity NT binding site" had long been considered as a mere "acceptor" site for NT, unrelated to any physiological significance [68]. NTS2 was cloned from three species: mouse [49], rat [15] and human [76]. However, probably based on this history, but also reinforced by its very unexpected properties, recognition of NTS2 as a true NT receptor has not been so easy to achieve.

3.1 One receptor, several cells, several independent pathways; one ligand, several efficacies:

Very few data are available on signal transduction pathways activated through stimulation of mouse NTS2. When this receptor was expressed in *Xenopus Laevis* oocytes, NT, neuromedin N, levocabastine and the NTS1 antagonist, SR 48692, triggered a Ca^{2+}-activated Cl^- current, suggesting coupling to $G_{q/11}$ in this experimental system [9,49]. However, upon expression of the receptor in HEK 293 cells, no alteration of cAMP, cGMP or InsP production was observed with either NT or levocabastine [8]. Similarly, no effect of NT was evidenced on InsP production when the receptor was expressed in COS cells [45]. SR 48692 was not evaluated in these last two studies.

Studies on the cloned human NTS2 [76] extended these first observations, further disclosing striking differences between NTS2 and NTS1. When this receptor was expressed in CHO cells, stimulation by SR48692 or another NTS1 antagonist, SR 142948A, increased InsP formation, calcium mobilization, arachidonic acid production and MAPK activity, but did not increase cAMP production [76]. In this study, neither the endogenous peptides, NT and neuromedin N, nor levocabastine exerted agonist activity, but instead antagonized the effects of the SR compounds. However, in a recent study using the same expression system [21], NT was found to increase activation of MAPK, with a lower maximal effect than SR 48692.

Another step was achieved through the demonstration that human NTS2 expressed in COS cells was constitutively active on InsP production [62]. SR 48692 retained agonist activity towards this transduction pathway, NT behaved as a neutral antagonist, and levocabastine as a weak partial inverse agonist [62]. When expressed in HEK-293 cells [30], human NTS2 constitutively activated serum response element (SRE) signaling through both pertussis-sensitive G proteins and Rho-GTPases, suggesting coupling to $G_{i/o}$ and $G_{12/13}$-type G proteins. Here again, NT behaved as a neutral antagonist on the constitutive signal.

By contrast with InsP production, human NTS2 expressed in COS cells was not constitutively active for Erk1/2 activation [30]. In these cells like in CHO cells, NT increased Erk1/2 activation. However, the NT effect was of small amplitude compared with those obtained for other receptors of the same family, such as ghrelin receptors [30]. SR 48692 was not evaluated in this work.

Very little is known about cloned rat NTS2 [15]. In a first study on this receptor expressed in CHO cells, SR 48692 and levocabastine, but not NT, were found to increase intracellular calcium [78]. However, it was further suggested that this effect of SR 48692 was NTS2-independent, since it also occurred in mock-transfected cells [21]. A similar rise in intracellular calcium by SR 48692 was also found on cultured rat cerebellar granule cells endogenously expressing NTS2 [66]. In these cells, no alteration of InsP production was observed following stimulation by NT, levocabastine, or SR 48692, and the SR 48692-induced calcium mobilization was suggested to occur through thapsigargin-sensitive calcium stores [66]. Moreover, like in CHO cells, and although cerebellar granule cells expressed NTS2, the SR 48692-induced calcium mobilization was suggested to be NTS2-independent, since it was not antagonized by NT or levocabastine. These data indicate that effects of SR 48692 on intracellular calcium should be interpreted with caution, since they may not always reflect interaction between this compound and NT receptors.

Stimulation of rat NTS2 expressed in CHO cells by NT, neuromedin N, levocabastine and SR 48692 increased Erk1/2 activation [21]. Like for the human receptor, which was investigated in the same study, the maximal Erk1/2 activation induced by NT was much lower than that induced by SR 48692. Interestingly, the SR48692 effect was not reduced upon addition of high concentrations of NT, leading to the proposal that the two agents might act through different receptor forms [21]. However, it could also be postulated that the NTS2-independent rise in intracellular calcium induced by SR 48692 in these cells might to some extent participate in the SR 48692-induced Erk1/2 activation, which could also account for the insensitivity of this effect towards NT application.

An NT-induced increase in Erk1/2 activation was also found on cultured rat cerebellar granule cells endogenously expressing NTS2 [66]. However, a major difference with the CHO cell expression system was that no increase in Erk1/2 activation was observed following application of SR 48692.

These data confirmed the coupling potential of NTS2 to diverse signal transduction pathways. They showed that the NTS1 antagonist, SR48692, mostly exerted agonist properties towards pathways associated with NTS2. They also indicated that the endogenous peptides NT and neuromedin N behaved as dual efficacy ligands, antagonizing SR-induced activation of several pathways while presenting agonist properties towards MAPK activation.

3.2 NTS2 signaling and G proteins: only one chapter of the full story?

Although many data are still lacking, these results may be tentatively interpreted in terms of NTS2-G protein interactions.

It can be suggested that human NTS2 may interact with $G_{q/11}$ [30,62,76], $G_{i/o}$ [30] and $G_{12/13}$ [30]. Whether interaction with $G_{i/o}$ might account for arachidonic acid release [76], as observed with NTS1 [53], remains to be established. By contrast, it seems that this receptor is not coupled to G_s, at least from the results obtained on CHO cells [76].

Coupling to $G_{q/11}$ remains to be confirmed for mouse NTS2, since SR 48692 was not tested in the previous studies in HEK and COS cells [8,45], and the question is still open for rat NTS2 [21,66]. Nothing is known on a possible interaction between these receptors and G_s, $G_{i/o}$ or $G_{12/13}$.

The constitutive activity of human NTS2 towards InsP production [62] and SRE signaling [30] suggested a ligand-independent activation of $G_{q/11}$, $G_{i/o}$ and perhaps $G_{12/13}$. It is not clear whether constitutive activity towards InsP production does not occur in CHO cells or was just missed in previous studies [76]. Interestingly, a ligand-independent increase in the Cl^- equilibrium potential had been observed following expression of mouse NTS2 in *Xenopus laevis* oocytes [49], suggesting that this receptor could also lead to constitutive activation of $G_{q/11}$ in this experimental system.

When compared to InsP production, both the lack of constitutive activity observed for Erk1/2 activation and the different efficacy exerted by NT provide a striking illustration of the independence between pathways already evoked for NTS1. However, a yet unanswered question is whether specific G proteins are involved in NTS2-mediated Erk1/2 activation. Indeed, several G protein-dependent or independent pathways may be involved in GPCR-mediated MAPK activation [43]. Erk1/2 activation by SR 48692 in CHO cells was not pertussis toxin-sensitive, and was inhibited by overexpression of dominant negative mutant of dynamin 1 [21]. Dependence of MAPK activation on dynamin-and/or β-arrestin was previously reported for other receptors, and was interpreted either as a permissive role of internalization [16,51] or, more recently, as a selective role of β-arrestins in signaling [73]. Interactions between NTS1 and β-arrestins were previously reported, involving serine and threonine residues in the receptor carboxyl-terminal tail [55], but no such interaction has been reported for NTS2. Moreover, whether dominant negative mutant of β-arrestin would also suppress Erk1/2 activation, similarly to the dynamin mutant, remains to be demonstrated.

Furthermore, are some G proteins necessarily involved in these processes? Recent data indicate that ligands endowed with inverse agonist activity towards the constitutive activity of "classical" G protein-dependent pathways may induce receptor-β-arrestin interaction and MAPK activation, like agonists [3]. However, by contrast with agonists, activation triggered by inverse agonists appears to occur through G protein-independent mechanisms [3]. In this respect, it would be interesting to evaluate whether such differential mechanisms apply to SR48692, NT and levocabastine, which all activate MAPK in CHO cells [21] but behave as agonist, neutral antagonist and inverse agonist, respectively, for constitutive InsP production in COS cells [62].

3.3 Conclusion:

These data indicate that NTS2 may interact with several G proteins. However, by contrast with the apparent constancy of the $G_{q/11}$-NTS1 receptor interaction from one species or one cell to another, no clear preference for a given G protein has yet emerged for NTS2. Going further along that line of thought, it might be revealed by future studies that the most consistently found signaling pathway is MAPK activation, and that this pathway does not involve any G protein, at least for NT itself. Moreover, further studies will be necessary to fully elucidate the species-, pathway- and cell-dependence of the constitutive activity observed up to now in some experimental systems, and also to identify the receptor domains involved in the interactions between NTS2 and the various G proteins.

It would also be interesting to reconsider the previously called "insensitivity of NTS2 agonist binding to guanine nucleotides" under the light of all available data. This property was previously identified using radolabeled NT as the radioligand. However, we presently know that NT acts as a dual efficacy NTS2 ligand and apparently exerts an agonist activity only towards MAPK activation, which may not involve any G protein. From the considerations developed above for NTS1, we also know that only some G proteins will alter the affinity of

some ligands, a property which might be related to the ability of the ligand to favor the formation of the receptor-G protein complex. Since SR 48692 and SR142948A appear as the most general NTS2 agonists towards $G_{q/11}$-, $G_{i/o}$- and perhaps $G_{12/13}$-related pathways, it would be interesting to investigate guanine nucleotide sensitivity of radiolabeled SR 48692 or SR142948A binding in different cells and receptor mutants allowing separated analysis of each receptor-G protein interaction. However, we can already expect that this property might appear to be unrelated to the ligand ability to activate the different signaling pathways.

4. Conclusion

Beyond its interest towards a better understanding of NT functions and design of new NTergic ligands with potential therapeutic applications, the study of G protein-NT receptor interactions emphasizes the wide diversity of signals which may arise from a single GPCR, depending on the cell and the ligand [26]. In this respect, NTS2 represents a striking illustration of the principle of parameter separation already evidenced for NTS1, at the receptor, pathway and ligand levels.

Whereas NTS1 readily appears as a *bona fide* NT receptor, this question has long been a matter of debate for the "levocabastine-sensitive NT binding site", and remains open due to the peculiar properties exhibited by the corresponding receptor, NTS2. However, although NT neither activates most NTS2-associated pathways nor alters constitutive activity when it occurs, it is now clear that at least one signaling pathway, MAPK activation, may be triggered by an interaction of NT with NTS2. This feature was observed not only in experimental expression systems but also in natural systems, such as cerebellar granule cells.

In that sense, there is no longer any problem to classify NTS2 as an NT receptor, which may mediate some functions of NT at a physiological level. Nevertheless, it remains possible that another endogenous ligand might exist for this receptor, and that some of its effects might resemble those displayed by SR 48692. Such a finding would further extend the

diversity of functional interactions and responses involving these receptors and our interest in devising appropriate models for their description.

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Legends for Figures.

Figure 1: EISAI-1 discriminates between pathways involving different receptor intracellular domains.

A: Interaction between NTS1 and $G_{q/11}$ involves the third receptor intracellular loop, linking transmembrane domains V and VI, while interactions with G_s and $G_{i/o}$ involve the receptor C-terminal tail following transmembrane domain VII. Whereas NT efficiently activated $G_{q/11}$, G_s and $G_{i/o}$, the NT(8-13) analog EISAI-1 ((Me)Arg-Lys-Pro-Trp-Tle-Leu-O-Et) mostly orientates the receptor towards the signaling pathways generated by the last two G proteins. This peculiar property of EISAI-1 is due to the esterification of its COOH terminus, preventing interaction with Arg327 in TMVI. A high affinity for NTS1 could be retained through anchoring of the ethyl moiety in the hydrophobic pocket formed by the transmembrane domains.

B: Illustration of concentration-response curves obtained for the NT(8-13) analogs JMV449 and EISAI-1 towards InsP production, Arachidonic acid release and cAMP production. When compared with JMV449 or other agonists not represented here (NT, neuromedin N or the unesterified analog of EISAI-1), EISAI-1 selectively presented both lower relative potency and efficacy to activate InsP production. Data taken from [69].

Figure 2: The cubic allosteric ternary complex model.

A: schematic representation of the model, with identification of some parameters relative to the action of the ligand (α) or the G protein (β, γ). Note the dual significance of γ. R, R*: generic terms for inactive and active receptor conformations, respectively [33].

B: partial representation of the extension of the model, in order to further discriminate between binding of R* to G and facilitation by R* of the G/G* transition (activation process). A single R* may present different efficacies to activate distinct G proteins. This phenomenon will be traduced by different values of the μ parameter.

Figure 3: Two ways of representing interaction between one receptor and two G proteins.

A: the bicubic model [33-35]. One active receptor state R* competes for the two G-proteins G1 and G2. A single agonist may present different parameter values for each pathway. Different agonists may stabilize different R*s, arising from the same or different Rs (hypothesis of multiple agonist-selective receptor states). This phenomenon will be traduced by different sets of parameters in the respective bicubic models generated for each agonist.

B: the three-state model [42]. Interaction with each G protein occurs through different receptor active states, R* and R**. A given agonist may differentially activate each pathway, based on its respective affinities for each receptor state. All agonists stabilize the same couple of active states R* and R**, although with different abilities (affinities). Here again, like in the bicubic model, formation of one receptor-G protein complex occurs at the expense of the other complex, due to the successive equilibria linking all receptor species.

Figure 4: Representing interactions between one receptor and two G proteins through isolated pathways.

A : Schematic representation of the « isolated pathway » mode in the cubic [33] and the three-state [42] models. This representation involves the same receptor forms or different receptor forms in each pathway.

B: Schematic representation of inversion of potencies between agonists among two signal transduction pathways. Due to the lower relative potency of one of the compounds towards pathway 2 (curves with closed triangles), different potency orders are observed among the two pathways. Although such a phenomenon was long attributed to the existence of multiple receptor subtypes, it can occur through stimulation of a single receptor linked to multiple pathways [33-35, 42, 69]. The bicubic model as well as the three-state model in their "intact mode", presented in Fig. 3, may accomodate differences in maximal effects ("intrinsic efficacies"). However, in the absence of receptor reserve for both pathways, only the description trough separate sets of equation, avoiding successive equilibria between species, may account for different relative potencies between pathways [42]. This situation may be due to particular conditions encountered in a given experimental system, such as compartmentalization of receptor species with G proteins in discrete membrane microdomains.