Master's thesis

Submitted as a document in partial fulfillment of the requirements for the degree of Master in Science

Title of project:

"CHARACTERIZATION OF THE CELLULAR AND MOLECULAR DETERMINANTS FOR THE INTERNALIZATION OF THE HIGH AFFINITY NEUROTENSIN RECEPTOR"

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July, 2002

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ACKNOWLEDGMENTS

I would like to thank the following people who made this Master's thesis a realization.

Dr. Alain Beaudet, for providing the brainchild of this project, as well as such an outstanding environment in which to carry it out. I am lucky to have been able to work in such a well-equipped, clean, and spacious laboratory. I am also grateful for all the editing that was done on earlier versions of the thesis, where his exceptional writing skills and creativity allowed my thesis to be of the quality it is today. Dr. Beaudet is an inspiration, especially with regards to his ambition and ability to keep it all together under moments of considerable stress.

Our secretary, Naomi Takeda, aka the "lab mom", for being such a great source of support and comfort, and feeding me healthy food! She is one of the most caring, lovely people I have ever come across.

Mariette Houle, who showed me how to perform every technique that was used in this work. Her patience was much appreciated, and I much admired her easy-going attitude and comforting words when things didn't go exactly as planned....

Anne Morinville, for her encouraging words and amazing ability to get things done. The initiative that she took on my behalf will never be forgotten. She is one of the most dedicated scientists I have ever seen, and am sure will be a roaring success in whatever her plans may be. Her editing was invaluable to the success of this Master's thesis.

Pierre Villeneuve, for being such a great person. His heart opens wide to friends in need, and I thank him for all the help he has given me over the past three years. He was truly a pleasure to work with, and I'm sure that his compassion and motivation will shine through throughout his medical career.

Drs. Cathy Cahill and Thomas Stroh, for sharing with me their knowledge and giving me some much needed support, and also for being such a pleasure to work with.

Dr. Phillipe Sarret, for his mischievious pranks and ability to lighten up my mood when it was much too intense, as well as his generosity and graciousness.

Minh Ky Nguyen, Mao Cheng Lee, Alex Choi Jackson, and Kelly McLellan, who provided me with confidence and motivation while they were around at the beginning of my project, and who ensured me that things do get better with time.

James Esdaile for just being James.

My advisors, Drs. Peter McPherson, who continues to astonish me with his drive and ability to excel at scientific discovery, and who generously provided me with many of the tools for my project, and David Ragsdale, probably the best "next door neighbor" I'll ever be lucky enough to have.

Monique Ledermann, the graduate department secretary, who continually goes above and beyond her call of duty to help out us students. The Neurology Department is very lucky to have such an extraordinary individual working for them.

Finally, I would like to thank my mother, who has dedicated her life to allow me to make something of mine, and my friends, who provided the day to day encouragement that has meant so much.

LIST OF ABBREVIATIONS

AP = Adaptor protein

AT1AR = Angiotensin receptor subtype 1A

B2-AR = Beta-adrenergic receptor subtype2

BSA = bovine serum albumin

CCK = cholecystekinin

CHO = Chinese hamster ovary

CSF = cerbral spinal fluid

DMEM = Dubelco's Modified Essential Medium

FBS = Fetal bovine serum

GFP = Green Fluorescent Protein

GFP-A-SH3 = GFP-tagged SH3 domain of amphiphysin2

GFP-I-EH = GFP-tagged EH domains of intersectin

GFP-INT = GFP-tagged full-length intersectin

GFP-I-SH3 – GFP-tagged SH3 domains of intersectin

GnHR = Gonadotropin hormone receptor

GPCR = G-protein coupled receptor

GRK = G-protein receptor kinase

HeLa = Helen Lane

IL-1 = interleukin 1

IL-8 = interleukin 8

KNRK = k-ras transformed rat kidney

m1, m2, m3, m4 = muscarinic receptor subtype 1, 2, 3, and 4, respectively

NTR = neurotensin receptor

RBL-2H3 = rat basophilic leukemia

SST1, SST2A, SST5 = somatostatin receptor subtype 1, 2A, and 5, respectively

TRH = thyrotropin-releasing hormone

TTBS = Transfection Tris-buffered saline

ABSTRACT

Neurotensin is a thirteen amino acid peptide that is found both in the central and peripheral nervous systems. In the CNS, it controls the synthesis and release of dopamine, modulates anterior pituitary hormone secretion, and provides non-opioid-receptor-dependent analgesia. In the gut, it modulates gastric acid secretion, pancreatic secretion, and colonic motility. In the immune system, it modulates IL-1 and mast cell secretion and stimulates macrophage phagocytosis. Neurotensin also acts as a growth factor for normal and cancer cells. Neurotensin mediates its effects through three neurotensin receptors: NTR1, NTR2, and NTR3. NTR1 and NTR2 belong to the G-protein coupled receptor (GPCR) family, while NTR3 is a single transmembrane receptor identical to sortilin. NTR1 has the highest affinity for neurotensin (Kd of 0.1-0.3 nM). Internalization of GPCRs, including NTR1 and NTR2, was shown to play an important role in initiating signaling activity as well as in desensitizing and resensitizing the receptors.

In this study, the internalization of the NTR1 was investigated. It had been shown previously that NTR1 internalizes via a time and temperature-dependent, clathrin-mediated pathway. In order to characterize some of the other molecular components required for the internalization of NTR1, COS-7 and HEK 293 cells were co-transfected with NTR1 and a dominant negative of either beta-arrestin, dynamin, intersectin, or amphiphysin, which have previously been documented to play a role in clathrin-dependent endocytosis. Transfected COS-7 and HEK 293 cells were assessed for NTR1 internalization in the presence or absence of these dominant negatives. The same study was repeated for the transferrin receptor, a nutrient receptor and does not belong to the GPCR superfamily.

The dominant negative dynamin K44A inhibited internalization of NTR1 and transferrin in both COS-7 and HEK 293 cells, while the dominant negative beta-arrestin V53D inhibited only the internalization of NTR1, and not transferrin, in both HEK 293 and COS-7 cells. The observation that the dominant negative of beta-arrestin inhibited NTR1 internalization and not transferrin internalization provides further evidence for the notion that beta-arrestin is involved specifically in the internalization of GPCRs, and not receptors belonging to other classes. Results also indicated that the amphiphysin2 dominant negative did not block the receptor-mediated internalization of either NTR1 or transferrin in either COS-7 or HEK 293 cells, suggesting that amphiphysin2 is not indispensable to clathrin-dependent-receptor internalization. By contrast,

intersectin inhibited internalization of NTR1 in COS-7 cells, but not HEK 293 cells, indicating that mechanisms of NTR1 internalization may differ depending on the cell type in which the receptor is expressed. Intersectin did, however, inhibit the internalization of the transferrin receptor in both COS-7 and HEK 293 cells, indicating that intersectin is clearly present in both cell types. Therefore, the dependence on intersectin of the internalization of NTR1 probably does not merely rely on the endogenous complement of intersectin in COS-7 and HEK 293 cells.

In summary, the results of the present work suggest that NTR1 internalizes in a beta-arrestin, dynamin-mediated pathway, with some dependence on intersectin (depending on the cell type in which it is expressed) and no dependence on amphiphysin2.

RESUME

La neurotensine (NT) est une tridecapeptide présente à la fois dans le système nerveux central et dans la périphérie. Au niveau central, la NT module l'activité des systèmes dopaminergiques, contrôle la libération d'hormones hypophysaires et induit une analgésie non antagonisée par la naloxone. En périphérie, ce peptide induit une inhibition de la sécrétion gastrique et régule la motricité gastrointestinale. Outre ces actions gastro-intestinale, la NT module la sécrétion d'IL-1, stimule la phagocytose par les macrophages et joue un rôle de facteur trophique au niveau des lignées cellulaires normales et cancéreuses. La NT exerce ses effets par interaction avec trois différents recepteurs NT : NTR1, NTR2, NTR3. Les deux premiers appartiennent à la famille des récepteurs à sept domaines transmembranaires couplés aux protéines-G (RCPGs), alors que le NTR3 est un récepteur à un seul domaine transmembranaire, également nommé sortiline. L'internalisation des RCPGs, dont les récepteurs NTR1 et NTR2 font parties, joue un rôle important dans la désensibilisation et la resensibilisation des récepteurs ainsi que dans l'initiation de nouvelles cascades signalétiques.

Le récepteur NTR1 présente une forte affinité pour la NT (kd = 0.1-0.3 nM) et internalise après stimulation par l'agoniste, via les vésicules tapissées de clathrine, de façon temps et température-dépendants. Dans le but de caractériser les autres partenaires impliqués dans l'endocytose du récepteur NTR1, ce dernier a été co-transfecté dans les cellules COS-7 et HEK-293 avec des protéines dominantes-négatives de la β-arrestine, la dynamine, l'intersectine et l'amphiphysine déjà connues pour leurs implications dans l'internalisation clathrine-dépendante. Le même type d'expérience a été effectué, en parallèle, en utilisant comme modèle d'étude, le récepteur de la transférine, n'appartenant pas à la famille des RCPGs.

Le mutant dominant-négatif de la dynamine K44A (dynamine K44A) bloque l'internalisation du NTR1 et du récepteur transférine dans les cellules COS-7 et HEK-293. Par contre, la protéine dominante-négative de la β-arrestine (β-arrestine V53D) inhibe uniquement l'endocytose du NTR1 dans les deux lignées cellulaires étudiées. Cette observation conduit à penser que la β-arrestine est seulement impliquée dans l'internalisation des RCPGs. Le mutant dominant-négatif de l'amphiphysine2 n'affecte ni l'internalisation du récepteur NTR1 ni celle de la transférine, indépendamment du type cellulaire étudié. Ces résultats suggèrent que l'amphiphysine n'est donc pas indispensable à l'endocytose clathrine-dépendant. La co-transfection du récepteur NTR1 avec le mutant dominant-négatif de l'intersectine bloque

l'internalisation du NTR1 dans les cellules COS-7 mais pas dans les cellules HEK-293, indiquant que le profil d'endocytose peut varier d'un type cellulaire à l'autre. L'intersectine peut cependant inhiber la séquestration de la transférine dans les deux lignées cellulaires, ce qui laisse suggérer que l'intersectine est présente dans les cellules COS-7 et HEK-293. La différence de profil d'internalisation du récepteur NTR1 observée dans les deux types cellulaires ne peut donc pas être le reflet d'un contenu endogène en intersectine variable entre les cellules COS-7 et HEK-293.

En conclusion, cette étude a permis d'établir que l'internalisation du récepteur NTR1 s'effectue selon un processus nécessitant le recrutement de la β-arrestine et de la dynamine. D'autre part, l'implication partielle de l'intersectine, conditionnée par le type cellulaire dans lequel le récepteur NTR1 est transfecté et l'absence de dépendance vis à vis de l'amphiphysine ont également été démontrées.

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Chapter 1- INTRODUCTION

1.1 G-Protein Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCRs) mediate the signal transduction of a wide variety of molecules, including hormones (i.e. somatostatin), neurotransmitters (i.e. serotonin and dopamine), inflammatory mediators (i.e. IL-1), neuropeptides (i.e. neurotensin), taste and odorant molecules, and even photons and calcium ions. Some events evoked by GPCRs are common to all cells. These include processes such as proliferation, differentiation, and ion homeostasis. In addition to these cellular processes, GPCRs in certain specialized cells mediate transduction of biological events unique to that cell type, such as chemotaxis for neutrophils, iodide uptake for thyrocytes, and aggregation for platelets. Almost without exception, GPCRs contain an extracellular amino (N) terminus, seven 20-25 hydrophobic residues that span the plasma membrane, three extracellular and three intracellular loops, and an intracellular carboxyl (C) terminal tail (see Figure 1). The only mammalian GPCR cloned to date not to have an intracellular C-terminal tail is the gonadotropin-releasing hormone receptor (GnRHR) (Heding et al, 1998).

The principle function of GPCRs is to transmit information about the extracellular environment to the interior of the cell. They do this by interacting with heterotrimeric G-proteins, which then activate various effector proteins that begin a signalling cascade that ultimately leads to a physiological cellular response.

The G-protein to which GPCRs are associated is composed of three subunits, called alpha, beta, and gamma. The binding of extracellular agonist induces GPCR activation and exchange of GDP for GTP on the alpha subunit of the cognate G-protein. This dissociates the alpha subunit from the beta-gamma subunits. The alpha and beta-gamma subunits go on to activate intracellular effector molecules which transmit the signal into the cell (Neer et al, 1995; Surya et al, 1998). Phosphorylation of specific serine and threonine residues in the receptor's C-terminal tail (Wang et al, 2000) by G-protein receptor kinases (GRKs) induces binding of beta-arrestin, which uncouples the receptor from its cognate G-protein and may initiate internalization of the ligand/receptor complex (desensitization and internalization may be dissociated events). Receptors are then dissociated from their ligands in endosomes, dephosphorylated, and either recycle back to the plasma membrane to mediate another round of signalling, are degraded in

lysosomes, or are targeted to specific regions of the cell. The series of events that take place in GPCR signalling are outlined in Figure 2.

1.1.1 Internalization of GPCRs

The concept that GPCRs are lost from the cell surface following agonist activation originated from the observation that beta2-adrenergic receptor (B2-AR) agonist treatment resulted in the loss of B2-AR recognition sites on the surface of frog erythrocytes (Chuang and Costa, 1979). Since then, a wide variety of GPCRs have been found to undergo agonist-stimulated internalization.

GPCRs enter the cell through receptor-mediated endocytosis. In the absence of agonist in the extracellular milieu there is a relatively slow rate of constitutive internalization of GPCRs from the cell surface. In the presence of agonist, however, the rate of endocytosis increases dramatically. Agonist-induced internalization has been observed for many GPCRs, including adrenergic receptors i.e. the alpha (Cowlen et al, 1998) and beta-adrenergic (Chuang and Costa, 1979) receptors, hormone receptors (i.e. thyrotropin releasing hormone (TRH) (Nussenzveig et al, 1993) and GnRH (Hazum et al, 1980)), chemokine receptors (i.e. CXCR1 (Prado et al, 1996) and CXCR4 (Signoret et al, 1997), neurotransmitter receptors (i.e. serotonin (5HT2A) (Berry et al, 1996) and dopamine (Ng et al, 1995)), olfactory receptors (Rankin et al, 1999), and peptide receptors, such as the NTR1 (Mazella et al, 1991).

Internalization of cell surface GPCRs is a critically important cellular process. In the normal, healthy organism, internalization of GPCRs allows the cell to respond to various agents, such as hormones or neurotransmitters, preventing the uncontrolled stimulation of cells. It also depletes the plasma membrane of high affinity receptors, which contributes to both the desensitization and resensitization of signalling. Desensitization is defined as "the loss or attenuation of a functional response despite the constant or prolonged presence of agonist", whereas resensitization is the term which refers to a receptor's recovery of a functional response after desensitization. By entering the endocytic pathway, the activated receptor is able to get dephosphorylated in endosomes and recycle back to the plasma membrane where it can again respond to agonist stimulation (resensitize). This is exemplified by the observation that the inhibition of B2-AR (Anborgh et al, 2000), neurokinin 1 (Southwell et al, 1998), and adenosine 2A receptor (Mundell et al, 1998) internalization prevents the resensitization of the receptor.

Internalization of muscarinic (Krudewig et al, 2000) and secretin receptors (Mundell et al, 1998) has been shown to be particularly important for their desensitization. The study of a receptor's internalization mechanism has also proven to be of use clinically as the accumulation of radiolabeled somatostatin agonists inside cancer cells that overexpress somatostatin receptors depends to a large extent on their receptor-mediated internalization through somatostatin type 2a receptors (SST2A) (Koenig et al, 1998).

1.1.1.1 Internalization of GPCRs occurs mainly through clathrin-coated pits

The predominant pathway for agonist-mediated internalization of GPCRs is via clathrin-coated pits. This mechanism involves a series of steps beginning with agonist binding to specific sites on the GPCR. The binding of agonist is thought to uncover endocytic signals in the C-terminal tail of the GPCR, in the form of tyrosine-containing stretches of amino acids or di-leucine motifs (Trowbridge et al, 1993; Letourner et al, 1994; Ohno et al, 1995; Rowe et al, 1996; Elrod-Erickson & Kaiser, 1996; Schmid et al, 1995). These endocytic motifs, such as YTRF in the transferrin receptor (where X is any amino acid) (Marks et al, 1997), bind to AP-2, the clathrin adaptor protein, which then recruits the receptor to regions that will eventually turn into clathrin-coated pits. AP2 also recruits clathrin triskelia to the plasma membrane (Keen et al, 1990), where they then polymerize to form a curved polygonal lattice that provides the mechanical scaffold for the coated pit, which causes subsequent curvature of the membrane. AP2 is believed to mediate clathrin binding to the membrane. It is located on the interior of clathrin lattices where it projects towards the vesicle membrane and binds to the membrane (Vigers et al, 1986). Clathrin binds with high affinity to membranes that have been stripped of their clathrin coat, but not when AP2 is removed (Mahaffey et al, 1990).

Agonist binding also stimulates GPCRs to undergo phosphorylation by specific serine/threonine kinases called G-protein Receptor Kinases (GRKs) (Bouvier et al, 1988). Subsequently, cytosolic proteins termed arrestins are recruited to the plasma membrane where they bind to agonist-occupied, phosphorylated receptors (Benovic et al, 1987). Arrestins also bind to AP2 (Laporte et al, 2000) and clathrin (Goodman et al, 1997), and helps to mediate the internalization of the GPCR through clathrin coated pits.

Once the clathrin-coated pit is formed, the GTPase dynamin is recruited to the plasma membrane either by amphiphysin (David et al, 1996) or intersectin (Hussain et al, 1999), where, with the help of amphiphysin (Takei et al, 2001), it severs the neck of the clathrin-coated pit through hydrolysis of GTP. The clathrin coat is rapidly shed after the vesicle has formed completely. The uncoating reaction is performed by an ATPase that is identical in sequence to the heat shock protein hsc70 (Schlossman et al, 1984; Greene et al, 1990). This ATPase has been shown to uncoat only vesicles that are fully formed, and not from invaginated coated pits that are still attached to the membrane. The internalization of a typical GPCR through clathrin-coated pits is diagrammed in Figure 3.

Clathrin-coated pits were first described by Roth and Porter in 1964 while studying mosquito oocyte uptake of yolk protein. These authors observed that while the cells were taking up the yolk, their plasma membranes invaginated, eventually forming vesicles, which were studded with spike-like projections. However, it wasn't until 1975 that the coat proteins were purified from bovine brain tissue by Pearse, and was found to be composed of the 180 kDa clathrin molecule.

The major unit of clathrin coated pits is the clathrin triskelion, which is a three-legged structure consisting of three heavy chains, of 190 kDa each, and three light chains which are tightly associated to the heavy chains (Kirchhausen et al, 1986; Ungewickell et al, 1981). The light chains come in two different types, LCa, about 23 kDa, and LCb, which is 27 kDa (Jackson et al, 1987). Associated with clathrin triskelia are adaptor proteins (APs). AP-2, which is specifically associated with the plasma membrane, is composed of four different subunits; two large subunits of about 100 kDa each, called alpha and beta, one medium chain of about 50 kDa called mu, and a fourth small chain of about 25 kDa, called omega. Structurally, AP2 has a brick like core, with the C-terminal of the two large subunits, alpha and beta, sticking out of the core like two appendages (Keen 1990; Pearse & Robinson, 1990; Brodsky et al, 1991; Kirchhausen, 1993; Payne, 1990). A diagram of a single clathrin triskelion, as well as an assembled clathrin cage, can be found in Figure 4.

The B2-AR (Moore et al, 1995), gastrin-releasing peptide receptor (Grady et al, 1995), TRH receptor (Drmota et al, 1998), thrombin receptor (Trejo et al, 2000), LH/HCG receptor (Bradbury et al, 1999), somatostatin type 2a receptor (SST2A) receptor (Boudin et al, 2000), platelet activating factor receptor (Le Gouill et al, 1997), angiotensin II type 1a receptor (AT1aR)

(Gaborik et al, 2001), olfactory receptor (Rankin et al 1999), adenosine 1A receptor (Navarro et al, 1999), m1, m3, and m4 muscarinic receptors (Vogler et al, 1999), dopamine D1 receptor (Gardner et al, 2001), neurokinin 1 receptor (Mann et al, 1999), cholecystekinin (CCK) receptor (Roettger et al, 1997), and the parathyroid hormone receptor (Ferrari et al, 1999) have all been shown to internalize via a clathrin-coated pit mediated pathway.

1.1.1.2 Caveolae

Although clathrin-mediated endocytosis mediates the internalization of most GPCRs, internalization of certain GPCR/ligand complexes has also been shown to depend on caveolae. Caveolae are flasked-shaped invaginations in the plasma membrane and are smaller in diameter than clathrin-coated vesicles (Palade 1953; Yamada 1955; Severs 1988). They have the ability to either internalize into the cell interior or to persist in the proximity of the plasma membrane (Kamen et al, 1988). The GTPase dynamin has been shown to be responsible for the budding of caveolae (Oh et al, 1998; Henley et. al, 1998; Schnitzer et. al, 1996). Caveolae are usually identified by the presence of the 21 kDa protein caveolin (Rothberg et al, 1992). Caveolae are rich in cholesterol and other lipids (Monier et al, 1996) and are found abundantly in epithelial cells (Peters et al, 1985) and in certain fibroblastic cells (Rothberg et al, 1992). Figure 5 illustrates the major components found in a caveolar pit.

GPCRs shown to be targeted to caveolae following agonist binding include CCK receptors in CHO cells (Roettger et al, 1995), M2 muscarinic receptors in cardiac myocytes (Feron et al, 1997), B2-AR in human epidermoid carcinoma A-431 cells (Dupree et al, 1993), and bradykinin (de Weerd et al, 1997) and endothelin (Chun et al, 1994) receptors in certain cell types. Up to 20% of CCK receptors are sequestered by caveolae after ligand binding, but when the coated pit pathway is blocked, nearly all receptors become sequestered by this pathway (Roettger et al, 1995). This illustrates that there is considerable plasticity in the cell regarding the mechanism of internalization used to internalize surface-bound receptors.

The goal of the present work is to define the specific molecular determinants required for the internalization of the high affinity neurotensin receptor (NTR1), in heterologous transfection systems, and to determine whether there is a difference in internalization pathways between two cell types, COS-7 and HEK 293.

1.2 Neurotensin

Neurotensin (NT) is a tridecapeptide (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) that was surrendipidously discovered and isolated in 1973 from bovine hypothalami during the course of the purification of substance P (Carraway and Leeman, 1973). Neurotensin got its name from its neuronal location and its ability to induce hypotension in the rat when injected *in vivo*. Early studies on neurotensin suggested it also had effects on the peripheral nervous system, as it was able to contract guinea pig ileum and relax rat duodenum (Carraway and Leeman, 1973).

Today it is appreciated that neurotensin is expressed both in the central and peripheral nervous systems, where it acts as a neurotransmitter and neurohormone, respectively. Administration of neurotensin in the central nervous system induces decreased locomotor activity (Kalivas et al, 1983) and hypothermia (Kalivas et al, 1985). NT's locomotor effects are thought to be mediated by neurotensin's interaction with the dopaminergic system (Nemeroff, 1980; Shi & Bunney, 1992). Central administration of neurotensin also induces non-opioid analgesia (Al-Rodhan et al, 1991) and changes in anterior pituitary hormone secretion (Rostene et al, 1997).

In the periphery, NT acts as a growth factor on a variety of normal (Feurle et al, 1987; Wood et al, 1988; Evers et al, 1992) and cancer (Davis et al, 1989; Iwase et al, 1996; Maoret et al, 1999; Seethalakshmi et al, 1997; Sehgal et al, 1994; Iwase et al, 1997; Yamada et al, 1995) cells. In the gut, it has been shown to modulate gastric acid secretion, pancreatic secretion, and colonic motility (Vincent et al, 1995). In the immune system, NT modulates interleukin-1 (IL-1) secretion (Lemaire et al, 1988), and stimulates macrophage phagocytosis (Goldman et al, 1982) and mast cell secretion (Carraway et al, 1982).

1.2.1 Neurotensin Receptors

Neurotensin's effects are mediated through its interaction with specific membrane-bound receptors. Three NT receptors have been identified: NTR1 (or NTRH, for the high affinity neurotensin receptor), NTR2 (or NTRL, for the low affinity neurotensin receptor), and NTR3. Two of these receptors, NTR1 and NTR2, are G-protein-coupled, and contain seven transmembrane spanning-domains connected together by three extracellular and three intracellular loops (Tanaka et al, 1990; Vita et al, 1993; Chalon et al, 1996; Mazella et al, 1996). NTR3 does not couple to G-proteins and has a single transmembrane spanning domain (Mazella et al, 1998).

The NTR1 mediates NT's high affinity binding sites (Kd for neurotensin binding to NTR1 is 0.1-0.3 nM (Kitabgi al, 1987)). NTR1 receptors are sensitive to sodium ions and GTP, both of which decrease the affinity of neurotensin for the receptor (Kitabgi et al, 1987). NTR1 also shows high affinity for the non-peptide antagonist SR 48692 (Gully et al, 1993). The use of the selective SR 48692 antagonist confirmed that neurotensin's effects on central dopaminergic pathways could be attributed to NTR1, and not NTR2, which has low affinity for this particular antagonist (Tanaka et al, 1990). The structure and amino acid sequence of the human NTR1 is depicted in Figure 6.

NTR1 couples to the G-protein Gq, which, upon agonist binding, initiates a signaling cascade that begins with the activation of phospholipase C (PLC) and ends in an increase of intracellular Ca, as illustrated in Figure 7. This cascade has been detected in rat brain (Goedert et al, 1984), neurons in primary culture (Weiss et al, 1988), cell lines that endogenously express NTR1 such as NIE-115 (Snider et al, 1986), NG108-15 (Imaizumi et al, 1989), and HT-29 cells (Amar et al, 1986), as well as in cells transfected with NTR1, such as CHO (Hermans et al, 1992) and LTK cells (Chabry et al, 1994).

Low affinity neurotensin binding is mediated by NTR2 (Kd = 3nM – 5nM) (Kitabgi et al, 1987). These sites are less sensitive to sodium ions than NTR1 and are insensitive to GTP (Kitabgi et al, 1987). The antihistamine drug levocabastine, which is devoid of any neurotensin-like pharmacological properties, can selectively block NT binding to NTR2 but not NTR1. The observation that the NTR1 specific antagonist SR48492 was unable to block a series of neurotensin effects including central analgesia and hypothermia suggest these effects could be mediated by NTR2 (Dubuc et al, 1994). As well, an increase in NTR2 mRNA in astrocytes of stab-wounded mice suggest the NTR2 might play a role in the regulation of inflammatory responses in retaliation to injury (Nouel et al, 1999).

NTR3, which is the most recently cloned receptor (Mazella et al 1998), is one of the rare peptide receptors that do not belong to the family of GPCRs. It has a single trans-membrane domain and is identical to the gp95/sortillin protein (Petersen et al, 1997). NTR3 has been shown to be mostly concentrated in intracellular compartments such as the endoplasmic reticulum and Golgi apparatus as evidenced from confocal microscopy studies (Mazella et al, 1998).

1.2.1.1 Internalization of NTRs

Numerous studies have indicated that NTR1 internalizes in an agonist-mediated, time and temperature-dependent fashion in both neuronal and non-neuronal cells.

1.2.1.1.1 NTR1 internalization in neurons

A study by Castel and colleagues in 1990 found that following intracerebral injection of I^{125}-NT in the rat neostriatum, the radioligand was retrogradely transported into the nigrostriatal pathway. Three lines of evidence suggested that the internalization and subsequent retrograde transport of the radioligand was receptor-dependent. First of all, ligation of neurons in the nigrostriatal pathway resulted in neurotensin receptor pile-up, indicating that both the ligand and receptor are internalized into the neuron. Second of all, this observation was abolished when non-radioactive NT1-13 or NT8-13 was co-injected into the neurons, but not when the NT1-8 was co-injected into the neurons. It has been previously established that the first 8 amino acids of the neurotensin peptide (NT1-8) do not bind to the NTR1 receptor, and do not mediate internalization or downstream signalling of the NTR1 (Vanisberg et al, 1991). Finally, the retrograde labeling of I^{125}-NT that was seen was selective towards nigral neurons, the projection of which were shown to presynaptically express the bulk of NTR1 receptors in the substancia nigra (Palacios et al, 1981; Quirion et al, 1985).

Another study which looked at NTR1 internalization in neurons was performed by Vanisberg et al in 1991. In this study, internalization of NTR1 was examined in primary cultures of rat neuronal cells. Results from this work showed that the binding sites for tritiated neurotensin decreased in a time and temperature dependent manner (no internalization was seen when neurons were kept at 0°C- 4°C) due to the internalization of NTR1. Internalization of the receptor was inhibited in the presence of bacitracin, which has been shown to inhibit the clustering of receptors into endocytic pits, an event which initiates endocytosis (Ray et al, 1989; Maxfield et al, 1979). The decrease in neurotensin receptor number was also seen in the presence of the active NT fragment NT1-13, but not in the presence of the inactive fragment NT1-8, suggesting the process was receptor-mediated.

Additionally, it was shown that iodinated NT internalizes within primary cultures of neurons derived from either embryonic mouse brain or SN-17 neuron-neuroblastoma hybrid cells, both of

which express high affinity NTR1 binding sites (Faure et al, 1992; Mazella et al, 1991; Checler et al, 1986). In these studies, autoradiographic experiments confirmed that after incubating at 37°C for 30 minutes, iodinated neurotensin was present inside neuronal cell bodies. At 10°C, a temperature which does not support NTR1 internalization, virtually no intracellular iodinated NT labeling was observed (Mazella et al, 1991).

Finally, a study using fluorescently tagged NT (fluo-NT) as a marker for NT and confocal microscopy directly demonstrated the internalization of NT in neurons. Cells in slices which bound the ligand at 4°C showed binding at the surface of the cell. When the cells were warmed up to 37°C for 45 minutes to facilitate receptor/ligand internalization, the signal from the surface of cells was dramatically decreased, while the cytoplasm became filled with red fluorescent granules, illustrating that NT internalization had occurred through a receptor-mediated mechanism (Faure et al, 1995).

1.2.1.1.2 NTR1 internalization in mammalian cells expressing NTR1

Numerous studies have been performed on the internalization of NTR1 using mammalian cell systems transfected with the NTR1. CHO cells stably transfected with a construct of NTR1 fused to GFP and exposed to a source of neurotensin internalized NTR1 in a time, temperature, and dose-dependent manner, as observed via confocal microscopy (Lenkei et al, 2000). HEK cells stably expressing the NTR1 were able to internalize 50-60% of surface-bound iodinated NT, a process that was inhibited upon incubation with hypertonic sucrose or PAO (Botto et al, 1998), agents that disrupt the formation of clathrin coated pits. Sucrose also inhibited internalization of NTR1 in transfected CHO cells, where internalization of this receptor has been found to occur normally at a rate of 90% (Hermans et al, 1994). Finally, a paper by Vandenbulcke et al (2000), which followed a tagged NTR1, demonstrated that NTR1 internalization in COS-7 cells was inhibited in the presence of hypertonic sucrose, cytosol acidification, and potassium depletion. These three agents have all been shown to inhibit the formation of clathrin-coated pits. This suggests that NTR1 internalizes via clathrin-coated pits, at least in COS-7 and HEK 293 cells. This study also demonstrated that NTR1 is targeted to lysosomes after internalization, while the NT ligand is recruited to a recycling endosome.

Mutagenesis studies conducted on the NTR1 demonstrated the importance of the carboxy-terminal (C-terminal) tail in internalization, as almost complete deletion of the

intracellular C-terminal site resulted in a near complete block in agonist-induced internalization (Chabry et al, 1995). Like most other GPCRs, the C-terminal tail of the NTR1 contains multiple serine, threonine, and tyrosine residues. Selective mutation studies revealed that the Tyr-422 and Thr-424 residues in NTR1's C-terminal tail are critically important for NTR1 internalization, as mutation of these two residues to glycine leads to a dramatic loss of NTR1 internalization (Chabry et al, 1995). The tyrosine is most likely required for the ability of NTR1 to bind to AP2 (Barak et al, 1994). The threonine, on the other hand, is phosphorylated by GRKs after agonist activation (Wang et al, 2000), leading to recruitment of beta-arrestin to the site of internalization.

It has also been shown that NTR1 directly binds to beta-arrestin1 and beta-arrestin2 (Oakley et al, 2000). Furthermore, the development of a GFP-tagged beta-arrestin construct has made it possible to visualize the translocation of the beta-arrestin molecule from the periphery of cells to intracellular vesicles upon agonist activation of a number of GPCRs. Agonist activation of NTR1 triggered a clear mobilization of beta-arrestin to the plasma membrane and subsequently intracellular vesicular compartments where it co-localized with internalized receptors (Zhang et al, 1999).

Therefore, present evidence strongly suggests that the internalization of NTR1 is mediated by a clathrin-dependent pathway and involves beta-arrestin. However, beta-arrestin has not been shown, to date, to be directly required for NTR1 internalization.

1.3 Components of the Endocytic Machinery

1.3.1 Beta-arrestin

After agonist stimulation, GPCRs are rapidly phosphorylated. This leads to the binding of arrestins which not only uncouple the receptor from its cognate G-protein but also directs it for internalization through clathrin coated pits.

The first arrestin, visual arrestin, was discovered while searching for the component that would completely quench signalling of the rhodopsin receptor (Pfister et al, 1985). Related molecules were sought that might terminate signalling of other GPCRs, primarily the B2-AR, and thus were found beta-arrestin1 (Lohse et. al, 1990) and beta-arrestin2 (Attramadal et al, 1992). Both beta-arrestin1 and beta-arrestin2 were found to be ubiquitously expressed in all tissues and,

when purified, were able to inhibit the signalling of phosphorylated B2-ARs by 75% (Lohse et al, 1990; Attramadal et al, 1992).

To date, the family of arrestins include 4 mammalian forms:

1. Visual arrestin, which is predominantly localized in rods and plays a role in inhibiting phototransduction (Kuhn 1978)
2. A retinal-specific arrestin, termed C or X-arrestin
3. Beta-arrestin1
4. Beta-arrestin2

Beta-arrestins 1 and 2 both play a role in the desensitization of B2-AR and other GPCRs.

Visual arrestins and C(X) arrestins are expressed mainly in the retina and pineal gland (Craft et al. 1994; Smith et al, 1994). The beta-arrestins are ubiquitously expressed outside the retina, but are predominantly located in neurons. Immunoelectron microscopy reveals that the beta-arrestins are concentrated at neuronal synapses along with GRKs (Arriza et al 1992; Attramadal et al 1992).

Mutations of the genes for Drosophila arrestins cause a defect in the ability of the animal to inactivate rhodopsin and results in retinal degeneration in about a week (Dolph et al, 1993). As well, in humans, a one base pair deletion in the arrestin gene is a cause of Oguchi disease, a congenital form of night blindness (Fuchs et al, 1995). More recently, it was determined that nephrogenic diabetes insipidus is due in part to a mutant vasopressin receptor that cannot signal. Specifically, the mutation allows the receptor to undergo internalization without agonist binding, and once internalized, it is unable to dissociate from beta-arrestin in endocytic vesicles. This prevents dephosphorylation and recycling of the internalized receptor. When the moieties on the receptor that bind beta-arrestin are eliminated, the vasopressin receptor is properly localized to the plasma membrane and can signal efficiently (Barak et al, 2001).

Arrestins preferentially bind to GRK-phosphorylated GPCRs as opposed to second messenger protein kinase phosphorylated receptors or non-phosphorylated receptors (Lohse et al, 1990; Lohse et al, 1992). The affinity of beta-arrestin binding to phosphorylated B2-AR receptors is 20-30 times higher than unphosphorylated receptors (Lohse et al, 1992).

Biochemical studies have shown that there is a direct interaction between beta-arrestin and clathrin triskelia. Mutagenesis studies have revealed that the clathrin-binding domain in beta-arrestin is localized to the C-terminal end of the molecule. Specifically, the clathrin binding domain in beta-arrestin has been localized to hydrophobic and acidic residues between amino acids 367 and 385 at its C-terminal end (Goodman et al, 1996). Correspondingly, the beta-arrestin binding domain on the clathrin molecule has been localized to amino acids 89 to 100 of its heavy chain (Goodman et al, 1997). Furthermore, beta-arrestin has also been shown to bind to the beta-adaptin subunit of the AP-2 clathrin adaptor protein complex, and overexpression of the region of beta-arrestin that binds to the beta subunit of AP2 inhibited the internalization of the B2-AR in HEK 293 cells (Laporte et al, 1999). Immunofluorescence in COS-1 cells determined that after agonist activation, B2-AR, beta-arrestin, AP2, and clathrin were all localized in the same regions of the cell (Laporte et al, 2000).

The receptor binding domain of beta-arrestin has been shown to be situated in the amino half of the arrestin molecule, which contains a small positively charged domain of about 20 amino acids which interacts with the GRK-phosphorylated C-terminus of GPCRs (Gurevich et al, 1995). Given that the beta-arrestin molecule has binding sites for GPCRs, clathrin, and AP-2, it can be readily appreciated that both beta-arrestin and AP-2 might function as adaptors that target GPCRs to clathrin-coated pits for internalization. However, the binding of beta-arrestin to the beta subunit of AP-2 is necessary for its targeting to the plasma membrane. Indeed, beta-arrestin2 mutants lacking the clathrin binding domain were still localized to the plasma membrane in HEK 293 cells, whereas mutants without the AP-2 binding motif were not (Laporte et al, 2000).

It has recently been discovered that the function of beta-arrestin in receptor internalization is mediated by its state of phosphorylation. Cytoplasmic beta-arrestin is constitutively phosphorylated on its C-terminal Serine 412 residue. Upon agonist-induced GPCR activation, cytoplasmic beta-arrestin is recruited to the plasma membrane where it rapidly becomes dephosphorylated. Dephosphorylation of beta-arrestin is required for clathrin binding and for the subsequent trafficking of the GPCR to clathrin-coated pits (Lin et al, 1997). This is underscored by the observation that a beta-arrestin mutant (beta-arrestin S412D), which mimics the phosphorylated form of beta-arrestin, when co-expressed into HEK 293 cells along with the B2-AR, dramatically inhibits B2-AR internalization. Furthermore, another beta-arrestin mutant which mimics the dephosphorylated form of beta-arrestin (beta-arrestin S412A), when expressed in HEK 293 cells with B2-AR, increases the extent of B2-AR internalization (Lin et al, 1997).

1.3.1.1 Beta-arrestins and their role in GPCR internalization

Beta-arrestin binding is specific for phosphorylated GPCRs as compared to other classes of phosphorylated receptors. Therefore, internalization of many GPCRs has been shown to be mediated by a beta-arrestin-dependent pathway. Table 1 summarizes the effects that occur when aberrant expression of beta-arrestin is artificially induced.

Although, as described above, there is considerable evidence that beta-arrestins mediate the internalization of GPCRs, some GPCRs have been shown to internalize in a predominantly arrestin-independent manner. For example, the internalization of the GnRHR was not affected by the co-expression of wild type or mutant beta-arrestin and did not induce translocation of beta-arrestin from the cytosol to the plasma membrane (Heding et al, 2000). The internalization of the secretin receptor was likewise unperturbed upon co-expression with the dominant negative V53D beta-arrestin mutant (Walker et al, 1999).

Interestingly, in the absence of overexpressed arrestins in HEK 293 cells, the m2 muscarinic acetylcholine receptor (m2) internalizes via an arrestin-independent pathway, as a beta-arrestin V53D dominant negative co-expressed with m2 in the same cells does not inhibit its internalization. If, however, arrestin2 or arrestin3 is co-expressed with m2 in HEK 293 cells, m2 can undergo arrestin-dependent internalization, and in fact its internalization increases three-fold. The dependence of this increase was confirmed to be dependent on arrestin as this increase in internalization is blocked by the V53D mutant of beta-arrestin (Pals-Rylaarsdam et al, 1997). Similarly, the internalization of the AT1aR was shown to be independent of beta-arrestin as assessed with studies using the V53D beta-arrestin mutant, but could be recruited towards an arrestin-dependent pathway upon overexpression of beta-arrestin with the receptor. This suggests that different endocytic pathways may be utilized in the same cell line, depending on the level of proteins expressed in the cell.

Intriguingly, activation of a chimeric AT1aR with the tail of a B2-AR causes beta-arrestin to remain at the periphery of cells, as it does when the wild-type B2-AR is stimulated, and activation of a chimeric B2-AR with an AT1aR tail causes beta-arrestin to translocate with the receptor deeper inside the cell, as seen when the wild-type AT1aR is activated (Anborgh et al, 2000). These results demonstrate that beta-arrestins are differentially regulated by the activation of distinct GPCRs, and that the C-terminal tail of the receptor might be involved in determining

the stability of arrestin/receptor complexes and thus the cellular distribution of beta-arrestins upon agonist activation.

The resensitization and recycling profile of a GPCR is related to the length of time to which it is bound to beta-arrestin. It is well-known that B2-AR resensitizes and recycles rapidly, while the V2 vasopressin receptor resensitizes slowly and recycles slowly (Pippig et al, 1995; Innamorati et al, 1998). For both receptors, beta-arrestin is involved in their internalization, but for the B2-AR, beta-arrestin dissociates from the receptor at the plasma membrane, whereas for the V2 receptor, beta-arrestins internalize into endosomes with the receptor. When the carboxy tail is switched between these two receptors, these effects are reversed (Oakley et al, 1999).

It has recently been shown that the NTR1 was able to interact directly with beta-arrestin 1 and beta-arrestin 2 with the same affinity (Oakley et al, 2000). As well, agonist activation of NTR1 was shown to translocate cytosolic beta-arrestin to the membrane where it co-localized with NTR1 (Zhang et al, 1999). Furthermore, while beta-arrestin dissociated from the dopamine and endothelin receptors at the plasma membrane, it remained with NTR1 even within the endosome (Zhang et al, 1999). This conforms to the hypothesis that receptors which do not readily recycle back to the plasma membrane, as demonstrated in the case of NTR1 (Botto et al, 1998; Vandenbulcke et al, 2000), remain associated with beta-arrestin within endosomes.

In this study, the dependence of NTR1 internalization on beta-arrestin was tested by using a dominant negative V53D mutant of beta-arrestin, which is a construct that binds well to clathrin, but has a very low affinity for phosphorylated GPCRs. Transferrin was used as a control, which was not expected to internalize in a beta-arrestin-dependent fashion, as it does not belong to the GPCR family of membrane proteins.

1.3.2 Dynamin

The 100kDa dynamin was first isolated in 1989 along with kinesin and dynein in calf brain microtubules where it was able to induce microtubules to slide past one another when stimulated with nucleotides. It was given the name dynamin, which means "power"in Greek (Shpetner et al, 1989). Despite dynamin's in vitro involvement in mediating microtubule sliding, (Schpetner et al, 1992), there was no strong evidence that dynamin stimulated microtubule movement through its hydrolysis of GTP.

A clue to dynamin's real in vivo function came when it was determined by van der Bliek et al in 1991 that the gene endocing the *shibire* allele in Drosophila was homologous to dynamin. The *shibire* allele encoded for a temperature-sensitive mutant protein that became inactivated at the non-permissive temperature. At the non-permissive temperature, Drosophila flies expressing the temperature-sensitive *shibire* allele become rapidly paralyzed (Grigliatti et al, 1973). This effect was reversible, as incubation at the permissive temperature allowed them to regain their normal phenotype. Upon close inspection of the axon terminals of these mutant flies, a striking depletion of synaptic vesicles was observed at the pre-synaptic membrane. Furthermore, the pre-synaptic membrane displayed deep invaginated pits, coated with an electron-dense material. It was therefore believed that these flies were defective in internalizing synaptic vesicles. The mutant phenotype could be rescued when the wild-type protein (i.e. the normal allele of *shibire*, which was homologous to dynamin) was inserted into the flies (van der Bliek et al, 1991). All other cell types that were examined in this Drosophila mutant showed an impairment in internalization, and therfore the effect seemed to be pleiotrophic (Poodry et al, 1973; Kessel et al, 1989; Narita et al, 1989). This was the first indication that dynamin could be involved in synaptic vesicle recycling and endocytosis in general.

1.3.2.1 Dynamin's mechanism of action

To determine dynamin's exact role in endocytosis, lysed nerve terminals were incubated with GTPγS, a non-hydrolysable GTP analogue, which would arrest endocytosis at the stage where hydrolysis of GTP was required. Similar in phenotype to the nerve terminals of the Drosophila *shibire* mutants, the lysed nerve terminals treated with GTPγS displayed deeply invaginated synaptic vesicles that were still attached by their necks to the plasma membrane. These extended necks were also encircled with electron-dense rings. These rings were evenly spaced and were identified with immunogold antibodies as dynamin (Takei et al, 1995). Additionally, experiments performed in mammalian cells using a GTPase-deficient dynamin mutant was shown to cause a block in internalization at the stage of invaginated pits, where the endocytic vesicles were still connected to the plasma membrane (Damke et al, 1994). Furthermore, dynamin, as well as a dynamin mutant deficient in GTP binding and hydrolysis (K44A), were able to form stacked rings in vitro in the absence of nucleotides (Hinshaw et al, 1995). This suggested that dynamin's mechanism of action was to pinch off the clathrin-coated pit to form a clathrin coated vesicle.

Direct evidence that dynamin could tubulate and vesiculate membranes came from a study in which purified dynamin, incubated with lipid extracts, was able to elongate them into tubules, even in the absence of nucleotides. These tubules resembled those that were formed in lysed nerve terminals when treated with GTPγS (Takei et al, 1995). Upon addition of 1 mM GTP, these tubules produced small vesicles that were of uniform dimension. In this same study, the K44A mutant dynamin was able to form tubes but could not vesiculate them in the presence of GTP (Sweitzer et al, 1998). Therefore, it was established that dynamin, by itself, could act to sever the necks of clathrin coated pits to produce clathrin-coated vesicles. Based on these observations, it has been suggested that dynamin may be the mediator of the budding off of clathrin-coated vesicles through a process requiring GTP hydrolysis.

A study by Stowell et al in 1999 provided evidence that dynamin's action may not be to pinch off vesicles, as was first believed, but rather to pop them off. When dynamin was incubated with lipid nanotubes, the characteristic tubules and electron-dense collars were formed. Before GTP hydrolysis, these dynamin rings were stacked tightly together. Upon addition of GTP, however, the distance between the spirals increased almost two-fold from 11 to 20 nm. This stretched the dynamin oligomers along the lipid tubules, whereas the diameter of the tubes did not change (Stowell et al, 1999). Thus it was suggested that perhaps dynamin pops off the vesicle by elongating the tubule until it breaks off. An extensive biophysical study of dynamin's mechanism of action by Kozlov et al predicts that the poppase model is probably the most accurate model of describing dynamin's mechanism of action (Kozlov et al, 1999).

The cellular event associated with GTP binding to dynamin was studied in 1995 by Baba and colleagues. The binding of GTP to dynamin was shown to be required for dynamin's redistribution from its uniform location around the perimeter of the clathrin-coated pit to the neck of the vesicle where it performs its mechanochemical action. HeLa cells transfected with either the GTP-binding deficient K44A mutant or a temperature sensitive mutant inhibited transferrin internalization. However, only the temperature sensitive mutants at the non-permissive temperature, which were not defective in GTP-binding and hydrolysis, showed the redistribution of dynamin around the neck of the coated pits from its original non-specific localization over the entire perimeter of the clathrin-coated pit; HeLa cells transfected with K44A, deficient in GTP binding, did not redistribute dynamin to its site of action (Baba et al, 1995). Furthermore, in permeabilized A431 cells, dynamin was found concentrated at the necks of coated pits, whereas the K44A mutant was evenly distributed on the clathrin coated pit in the same arrangement to

when it binds flat lattices (Damke et al, 1994). Finally, redistribution of dynamin to the necks of coated pits was inhibited when incubated with GDPβS, which forces dynamin to remain in its GDP-bound state (Warnock et al, 1997). Therefore, GTP binding is required to promote the redistribution of dynamin to the correct location on the clathrin coated pit, while GTP hydrolysis is required for scission of the coated pit into coated vesicles.

1.3.2.1 The Dynamin Family

Dynamin is expressed by three separate genes, which code for dynamin1, dynamin2, and dynamin3. Dynamin1 is expressed mainly in neurons (Scaife & Margolis, 1990; Powel & Robinson, 1995) and has 8 different splice variants (Cao et al, 1998). Dynamin2 is ubiquitously expressed (Cook et al, 1994; Sontag et al, 1994; Diatloff-Zito et al, 1995), has 4 different splice variants, and has been mapped to human chromosome 9 (Newman-Smith et al, 1997). Dynamin3 is expressed in the testes (Nakata et al, 1993), brain, and lung (Cook et al, 1996) and has 13 different splice variants (Cao et al, 1998).

Different dynamin isoforms are found in different tissues and cell types, allowing dynamin to mediate a general role in vesicle budding throughout the organism. For instance, dynamin1 is mainly found in neurons, whereas dynamin2 is ubiquitously expressed. The different splice variants of these genes may allow for specific targeting of a particular isoform within the cell. For example, the dynamin2 splice variant Dyn2aa localizes both to the plasma membrane and Golgi apparatus, while the dynamin2 splice variant Dyn2ab is restricted exclusively to the plasma membrane (Cao et al, 1998).

1.3.2.2 Dynamin's domains

Dynamin contains several domains that each perform a different function in mediating its role of budding off clathrin-coated vesicles (Figure 8).

At its N-terminal, between amino acids 1-299, lie three GTP-binding domains, within which dynamin performs its GTPase activity. The next string of amino acids, from 300-520, comprises dynamin's middle domain, which contains a coiled-coil region that is involved in dynamin's self-assembly into oligomers (Smirnova et al, 1999; Okamoto et al, 1999). The PH

domain of dynamin runs from amino acids 521-622 and binds to the βγ subunit of G-proteins. This interaction inhibits dynamin's GTPase activity, and is probably involved in dynamin's ability to mediate the endocytosis of GPCRs. The PH region also targets dynamin to membranes by binding to phosphoinositides. If the region in the PH domain that binds phosphoinositides is deleted and transfected into COS-7 cells, transferrin internalization is inhibited (Achiriloaie et al, 1999). COS-7 cells (Vallis et al, 1999) and HeLa cells (Lee et al, 1999) transfected with dynamin mutants lacking their PH domains exhibited a defect in transferrin receptor internalization.

Dynamin's GED (GTPase Effector Domain) is located between the proline-rich domain (PRD) and PH domains at amino acids 623-745. It interacts directly with the GTPase domain to stimulate its GTPase activity. Removal of the GED domain renders a dynamin that still has the ability to bind GTP but whose rate of GTP hydrolysis is severely reduced (Muhlberg et al, 1997). The GED domain is able to stimulate dynamin's GTPase activity by 50-100-fold upon self-assembly of dynamin (Muhlberg et al, 1997). This domain is also responsible for recognising other dynamin isoforms. Dynamin1 and dynamin2 can be co-immunoprecipitated from nerve terminals indicating that it's possible for them to bind to one another *in vivo* (Okamoto et al, 1999). Dynamin's C-terminal domain houses the last string of amino acids of the molecule (746-864). Its C-terminal region is rich in proline residues and contains several SH3 binding sites. Dynamin binds to many proteins through its C-terminal domain, many of which are important in endocytosis. Among these are intersectin and amphiphysin. Dynamin also binds Ca^{2+} and is phosphorylated and dephosphorylated at its C-terminus. Dynamin's Ca^{2+} binding and state of phosphorylation regulate dynamin's activity at the nerve terminal. Upon nerve terminal depolarization and Ca^{2+} binding, dynamin is dephosphorylated by calcineurin and is targeted to the plasma membrane. In the resting nerve terminal, dynamin is phosphorylated by phosphokinase C (PKC), which inhibits its ability to bind to lipids, and thus stays sequestered in the cytosol. This is one way dynamin is prevented from vesiculating all cell membranes.

1.3.2.3 Dynamin's binding partners

Dynamin's binding partners suggest that this protein is intamitely involved in receptor-mediated internalization. Dynamin co-localizes with clathrin and binds to the appendage domain of alpha-adaptin in the AP-2 molecule (Wang et al, 1995; David et al, 1996). It also binds to the SH3 domain of amphiphysin (David et al, 1996), as well as to the SH3 domains of intersectin which have been implicated in synaptic vesicle recycling and receptor-mediated endocytsosis.

1.3.2.4 Dynamin's role in other cellular processes

In addition to dynamin's role in vesicle budding at both synaptic terminals and at the plasma membrane of most cell types, dynamin has been implicated in a number of other cellular processes, such as:

1. Determining cell shape and mediating cellular movement (Damke et al, 1994; McNiven et al, 2000).

2. Budding of secretory vesicles from the Golgi complex (Jones et al, 1998).

3. The budding of caveolae (Oh et al, 1998; Henley et al, 1998; Schnitzer et al, 1996).

4. The internalization of the GLUT4 glucose transporter (Volchuk et al, 1998).

5. The internalization of the adenovirus, Semlike Forest virus, and VSV (Marsh et al, 1980; Superti et al, 1987).

6. The mediation of phagocytosis in macrophages (Gold et al, 1999).

7. The internalization of ionotropic AMPA receptors (Carroll et al, 1999).

8. Regulation of p53-mediated apoptosis (Fish et al, 2000). Dynamin2, when overexpressed in either HeLa cells, U373 microglial cells, rat fibroblasts, or primary fibroblasts, is extremely toxic and leads to cell death. This is due to dynamin2's ability to activate the transcription factor p53, which, upon entry into the nucleus, initiates a cascade of events that end in either arrest in the cell cycle or apoptosis. More than 50% of cancers contain a mutation in the gene encoding p53 (Levine et al, 1997). This effect is exclusive to dynamin2, as even 200-fold overexpression of dynamin1 fails to have an effect on cell viability (Fish et al, 2000).

1.3.2.5 Dynamin's Role in GPCR endocytosis

Once a clathrin-coated pit is formed by clathrin and AP2, dynamin gets recruited to the plasma membrane, perhaps by amphiphysin or intersectin. At the plasma membrane, it binds GTP, is directed to the necks of clathrin-coated pits, and pops off the vesicle through GTP hydrolysis. At the site of the clathrin-coated pit, dynamin also binds to the βγ subunits of G-proteins through its PH terminal, which inhibits its GTPase activity. This allows the accumulation of GTP-bound dynamin near the necks of the coated pit. When the concentration of dynamin oligomers is at a critical value, this inhibition is released, allowing GTP-hydrolysis and release of the clathrin-coated vesicle. This is a regulatory mechanism that is in place to prevent dynamin's potentially uncontrollable ability to sever all cell membranes.

Dynamin has been implicated in mediating the internalization of many GPCRs (see references in Table 2). However, as indicated in this table, there are some receptors, that, when overexpressed with a dynamin mutant in certain cell types, are not affected in their ability to undergo agonist-mediated internalization. These include the secretin receptor (Walker et al, 1999), the dopamine D2 receptor (Vickery et al, 1999), the m2 muscarinic receptor (Schlador et al, 2000), and the AT1AR (Zhang et al, 1996). Table 2 summarizes the studies that have looked at dynamin's role in the internalization of various GPCRs.

It is not yet known whether NTR1 internalizes in a dynamin dependent or independent fashion. In this study, a dominant negative K44A mutant dynamin was transfected into both COS-7 and HEK 293 cells to see what effect it had on NTR1 internalization. COS-7 and HEK 293 cells were also transfected with K44A alone to assess the internalization of transferrin, which served as a control, since it has been previously shown that transferrin internalizes in a dynamin-dependent fashion.

1.3.2 Amphiphysin

Amphiphysin was first identified by screening a chicken brain cDNA library with antibodies raised against synaptic proteins. It is localized mainly to presynaptic nerve terminals (Lichte et al, 1992), but has also been found at low levels in testes, lung, muscle, and fibroblasts (Herscovits et al, 1993).

Amphiphysin's molecular structure can be broken down into three domains. At its C-terminal end, it contains a single SH3 binding site that binds to a proline-rich sequence at the C-terminus of dynamin, encoded by the amino acids PSRPNR. This SH3-binding site on amphiphysin is located between amino acids 833 – 838 (Folli et al, 1993; De Camilli et al, 1993). The interaction of dynamin's proline-rich region with amphiphysin's SH3 domain has an affinity of 190 nM, making it one of the strongest SH3 interactions known (Grabs et al, 1997). Amphiphysin's central domain contains two sequences of amino acids that allow it to bind to clathrin and AP2. The region encoding for amino acids 322-340 of amphiphysin allows it to interact with the alpha subunit of AP2 (Wang et al, 1995; David et al, 1996), and the region that codes for amino acids 347-386 mediates its binding to clathrin itself (McMahon et al, 1997). Amphiphysin also contains an N-terminal coil-coil region that allows it to form heterodimers and which is responsible for targeting it to the plasma membrane (Slepnev et al, 1998).

The second amphiphysin gene (amphiphysin2) in mammals was cloned by eight different groups (Wigge et al, 1997; David et al, 1996; Sakamuro et al, 1996; Sparks et al, 1996a,b; Leprince et al, 1997; Ramjaum et al, 1997; Butler et al, 1997). Like amphiphysin1, amphiphysin2 binds to dynamin, AP-2, and clathrin on independent sites and is localized mainly to the brain (Ramjaun et al 1997). In the brain, amphiphysin2 shares a similar distribution to amphiphysin1, being highly concentrated in nerve terminals (Wigge et al, 1997; Ramjaun et al, 1997). Plasma membrane targeting is dependent on the presence of a 31 amino-acid sequence at the N-terminus of amphiphysin2. The N-terminus of amphiphysin1 can bind to both amphiphysin1 and amphiphysin2, and mediates formation of homo- and heterodimerization (Slepnev et al, 1998). COS-7 cells transfected with either amphiphysin1 or amphiphysin2 inhibited transferrin internalization (Wigge et al, 1997), but this inhibition was reversed if the cells were transfected with both isoforms. It is therefore believed that the functional unit of amphiphysin in endocytosis is its dimerized form. Both SH3 domains in the heterodimer are able to bind dynamin, and when bound to dynamin, the amphiphysin1-amphiphysin2 heterodimer stimulates its GTPase activity (Wigge et al, 1997).

Interestingly, it was found that many splice variants of amphiphysin2 exist, some of which are expressed in a tissue-specific manner (Wigge et al, 1997; Ramjaun et al, 1997; Leprince et al, 1997; Sparks et al, 1996; Sakamuro et al, 1996; Butler et al, 1997: Tsutsui et al, 1997; Kadlec et al, 1997). In fact, it turns out that amphiphysin2 is ubiquitously expressed although its subtype composition shows a significant tissue specificity (Tsutsui et al, 1997).

Given amphiphysin's ability to bind multiple components of the endocytic machinery, its role in this process was investigated. Evidence that amphiphysin is involved in clathrin-mediated internalization in both neuronal and non-neuronal cells include the following:

1. Amphiphysin knock-out mice were shown to have decreased synaptic vesicle recycling efficiency (Di Paolo et al, 2002).

2. The SH3 domain of amphiphysin1 blocks receptor-mediated endocytosis of the EGF and transferrin receptor when transfected into COS-7 cells (Wigge et al, 1997).

3. Transfection of the clathrin and AP2 binding sites of amphiphysin1 comprising amino acids 250 to 588 in CHO cells acts as a dominant negative, blocking receptor-mediated uptake of transferrin and changing the staining pattern of clathrin from the typical punctate to a diffuse pattern, consistent with a disruption in clathrin assembly (Slepnev et al, 1998).

4. Microinjection of the amphiphysin1 SH3 domain inhibited synaptic vesicle endocytosis at the stage of invaginated coated pits. The SH3 domain of amphiphysin1, when injected presynaptically into the lamprey giant reticulospinal synapse, creates an accumulation of invaginated endocytic pits (Shupliakov et al, 1997).

5. Microinjection of the amphiphysin1 SH3 domain prevents transferrin and GLUT4 internalization in 3T3-L1 fibroblasts and adipocytes (Volchuk et al, 1998).

6. Co-localization of amphiphysin and dynamin in many brain regions was detected via double immunofluorescence, and significant amounts of dynamin can be co-precipitated with amphiphysin from brain extracts (David et al, 1996).

7. Adding the SH3 domain of amphiphysin to cultured hippocampal neurons disrupted internalization of GABA receptors (Kittler et al, 2000).

8. Senescent cells have been shown to be inhibited in receptor-mediated endocytosis. In a study by Parks and colleagues in 2001, an impairment of receptor-mediated transferrin endocytosis in senescent cells was determined to be due to a selective decrease in the amount of amphiphysin, but not dynamin or AP-2. Furthermore, the reduced rate of transferrin

internalization exhibited by senescent cells could only be rescued when full length amphiphysin, but not dynamin or AP-2, was injected into the cells.

1.3.2.1 Amphiphysin's mechanism of action

Surprisingly, it was shown that amphiphysin by itself could transform liposomes into tubules to a greater extent than the same concentration of dynamin, and that it was assembled with dynamin in rings around GTPγS treated nerve terminals. In the presence of amphiphysin, dynamin's fragmenting ability was enhanced. It was determined that the N-terminal fragment of amphiphysin, specifically amino acids 1-286, was sufficient for it to mediate tubulation of the liposomes (Takei et al, 1999). As well, the tubules were stained with immunoreactivity to amphiphysin. These observations suggest that amphiphysin does not only act as a passive binding partner of dynamin by recruiting it to the membrane, but very likely participates in the fission reaction itself.

The SH3 domains of amphiphysin1 and amphiphysin2, unlike all other SH3 domains, favor the disassembly of dynamin rings by sequestering the dissociated form (Owen et al, 1998). Therefore, in order for endocytosis to occur, dynamin has to detach from amphiphysin so it can form oligomers and bind to synaptic vesicle membranes. It is thought that amphiphysin recruits dynamin monomers to the sites of clathrin-coated pits on the plasma membrane, thus preventing their premature assembly away from clathrin-coated pits. When the dynamin/amphiphysin complex reaches clathrin-coated pits at the plasma membrane, amphiphysin can bind to clathrin and AP2, and in so doing decreases dynamin's binding affinity either via a conformational change in amphiphysin upon binding clathrin or due to stearic hindrance. It has been shown that when clathrin binds to amphiphysin, dynamin's binding is decreased, and vice versa; in the presence of a high concentration of dynamin, clathrin binding is inhibited. When a critical amount of clathrin has bound to amphiphysin, dynamin, having a lower affinity for amphiphysin, is released at the site of clathrin-coated pits. At this point, along with amphiphysin, dynamin can oligomerize around the necks of synaptic vesicles and in doing so, stimulate its GTPase activity, which could produce the energy required to mediate the fission of clathrin-coated pits into detached synaptic vesicles.

Amphiphysin has also been shown to mediate phagocytosis along with dynamin, (Gold et al 2000), and a splice variant of amphiphysin 2, BIN1 has properties of a tumor suppressor and is underexpressed in a variety of human cancer tissues (Sakamuro et al, 1996).

As amphiphysin's SH3 domain, when overexpressed in cells, tends to have a dominant negative effect on clathrin-mediated internalization, and particularly on the internalization of EGF and transferrin receptors, the putative role of amphiphysin in NTR1 internalization was investigated in the present study. The SH3 domain of amphiphysin2, tagged to GFP, was transfected into COS-7 and HEK 293 cells and the effect on both transferrin and NTR1 internalization was assessed. At the time of this writing, this is the first time that amphiphysin2 has been studied for its role in the internalization of a GPCR.

1.3.3 Intersectin

Intersectin was isolated in 1998 by Yamabhai et al and is composed of two N-terminal EH (Eps15 homology domains) domains, a central coiled-coil region, and five SH3 (Src homolgy 3) domains at its C-terminus (Figure 9). EH and SH3 domains have been shown to be able to form protein-protein interactions with other molecules, many of which are known to be involved in endocytosis. Thus intersectin derives its name from the potential for it to bring together many components of the endocytic machinery to form a complex and to target it to the sites of clathrin-mediated endocytosis.

SH3 domains are anywhere from 50-70 amino acids long and recognize motifs rich in proline, such as the PXXP motif. The presence of multiple SH3 domains (SH3 A-SH3 E) in intersectin suggested that it might bind to dynamin, as dynamin has a proline-rich domain at its C-terminus. Indeed, it was shown that dynamin binds very strongly to the SH3 C, SH3 D, and SH3 E motifs, and with less affinity to SH3A and SH3B domains of intersectin (Okamoto et al, 1999).

EH domains are approximately 100 amino acids long and were first detected in a protein called Eps15,which is a substrate for the epidermal growth factor receptor tyrosine kinase (Wong et al, 1995). Eps15 has three EH domains and binds to AP2 via its C-terminal domain (Benmerah et al, 1996). HeLa cells transfected with an Eps15 mutant lacking the second and third EH domains redistributes the punctate, plasma membrane coated-pit localization of AP-2, clathrin, and dynamin to a homogenous cytosolic one and inhibits transferrin internalization (Benmerah et

al, 1999). Intersectin was found to co-localize with Eps15 staining in COS-7 cells (Pucharcos et al, 2000) and forms heterodimers through its coiled-coil region with Eps15 (Sengar et al, 1999).

This suggests that EH domains play a role in targeting components of the endocytic machinery to the plasma membrane. Intersectin's EH domains, when expressed by themselves, co-localize with clathrin in COS-7 cells, suggesting that it is indeed the EH domain of intersectin that targets the intersectin molecule to clathrin-coated pits (Hussain et al, 1999).

EH domains preferentially bind to NPF (asparagine-proline-phenylalanine) motifs. In addition to Eps15, intersectin binds to two other proteins involved in endocytosis. One is SCAMP (secretory carrier membrane proteins), which contains multiple NPF motifs, and is an integral membrane protein that's found ubiquitously in all cells (Brand et al, 1991; Singleton et al, 1997). COS-7 cells overexpressing SCAMP or transfected with a dominant mutant SCAMP which lacks its NPF N-terminal region blocked the internalization of transferrin (Fernandez-Chacon et al, 2000). Another protein that binds to intersectin's EH domain is stonin2, which is a protein that is ubiquitously expressed in all cells and contains NPF motifs. Overexpression of stonin2 in HeLa cells interferes with AP2 recruitment to the plasma membrane and impairs the internalization of the EGFR, transferrin, and LDL receptors (Martina et al, 2001).

Intersectin is endogenously expressed in COS-7 and PC12 cells, and has been shown to colocalize with clathrin in COS-7 cells and hippocampal neurons (Hussain et al, 1999). Additionally, antibodies towards intersectin immunoprecipitate clathrin, AP2, and dynamin from rat brain synaptosomes (Hussain et al, 1999).

Two forms of intersectin exist due to alternative splicing. One is a ubiquitously expressed form, and another is brain specific. This latter form contains the two EH domains, coil-coil region, and five SH3 domains, in addition to a guanine-nucleotide exchange factor (GEF) domain, a pleckstrin homology (PH) domain), involved in phospholipid binding, and a C2 domain (Guipponi et al, 1998), which has been shown to be involved in Ca^{2+}-dependent phospholipid binding. These last two regions indicate that this form of intersectin might have the ability to bind membranes, and may anchor the endocytic complex near sites of clathrin-coated pits.

These data strongly implicate intersectin as a component of the endocytic process. It was a goal of this project to determine whether intersectin was involved in the internalization of the

GPCR NTR1, and to compare that to the effect it has on the internalization of the transferrin receptor, which does not belong to the GPCR family.

1.4 The Transferrin Receptor

Iron is a nutrient that is required by every living cell. Transferrin is a ubiquitously expressed serum glycoprotein that transports iron to all tissue cells from the liver, which is the main storage site of iron in the body, to the intestine, the site of iron absorption. Transferrin associates with plasma membrane receptors in a di-ferric state. The iron free form is apotransferrin, and it binds two iron atoms very tightly to form ferrotransferrin. The association of iron with transferrin is stable at the pH of serum but is unstable at lower pH. The iron therefore dissociates from transferrin once internalized and is retained for use by the cell. Transferrin remains bound to the receptor at low pH, and the receptor-ligand complex recycles back to the plasma membrane. At the neutral pH of the extracellular environment, apotransferrin (transferrin without iron) dissociates from its receptor and is free to bind to iron and to repeat the cycle again. Figure 10 diagramatically illustrates the transferrin receptor's internalization and recycling pathway.

Transferrin is a single transmembrane protien that binds iron from the blood and brings it into the cell. The transferrin receptor, once delivery of the iron has occurred, then recycles to the plasma membrane. The receptor also internalizes constitutively, that is, without it having to bind transferrin. This is very different from the NTR1, which spans the plasma membrane seven times, acts as a neurohormone and neuromodulator, and which couples to G-proteins to send a message into the cell. NTR1 does not recycle and has a very low rate of constitutive internalization (Botto et al, 1998). The transferrin receptor has been shown to internalize via a clathrin-mediated pathway in many types of cells (van Dam et al, 2002; Wu et al, 2001; Buss et al, 2001).

1.5 Thesis objectives

In summary, the objective of the present study was to assess the dependence of NTR1 internalization on four proteins that have been shown to be involved in receptor-mediated internalization:

- Beta-arrestin
- Dynamin
- Amphiphysin2
- Intersectin

To this end, two cell lines, COS-7 and HEK 293, were co-transfected with NTR1 and a dominant negative of one of these four proteins, and the ability of each cell line to internalize NTR1 was assessed (see Figure 11 for summary diagram). The transferrin receptor was studied in parallel to determine if both types of receptors, i.e. a GPCR (NTR1) and a single-transmembrane, constitutively internalized receptor (transferrin), require similar components of the endocytic machinery to internalize into the cell. As well, the studies on the transferrin receptor served as an internal standard to confirm that the methodology used to assess receptor internalization was appropriate (see Figure 12).

Figure 1 – Schematic diagram of a typical G-protein coupled receptor. GPCRs are characterized by seven transmembrane domains (TM1-TM7), three extracellular (EC1-EC3) and three intracellular (IC1-IC3) loops, an extracellular N-terminus, and an intracellular C-terminus.

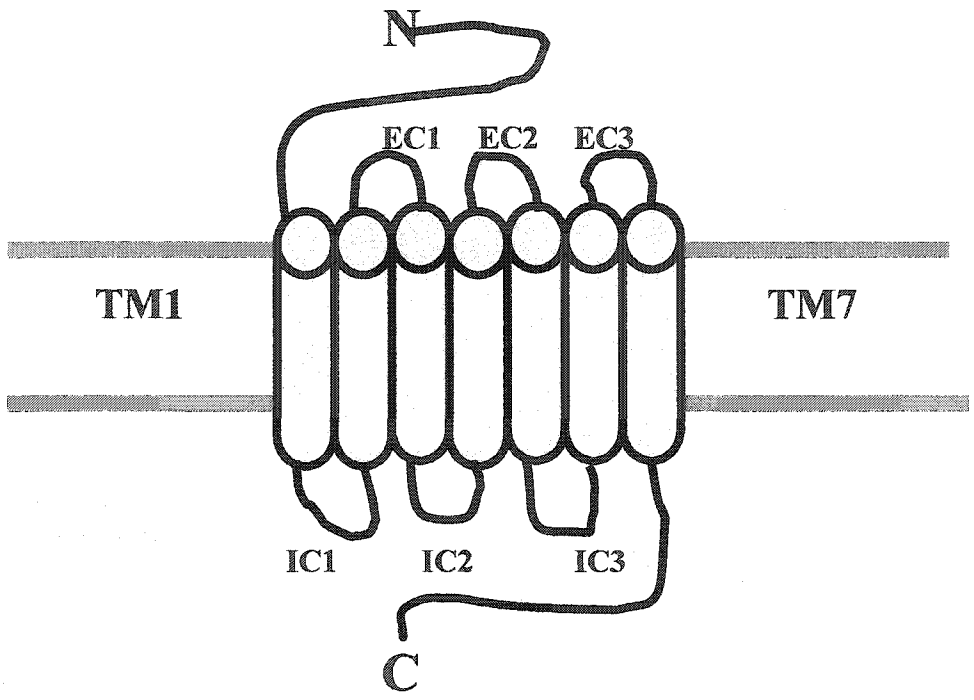
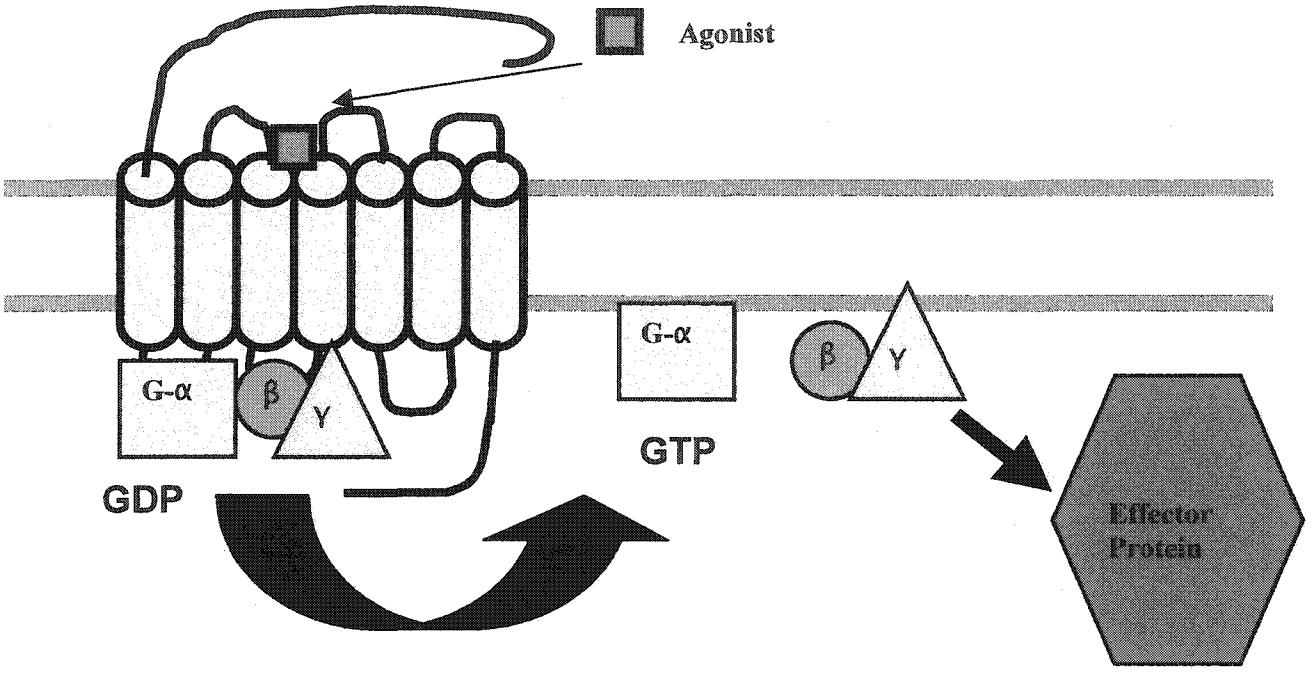


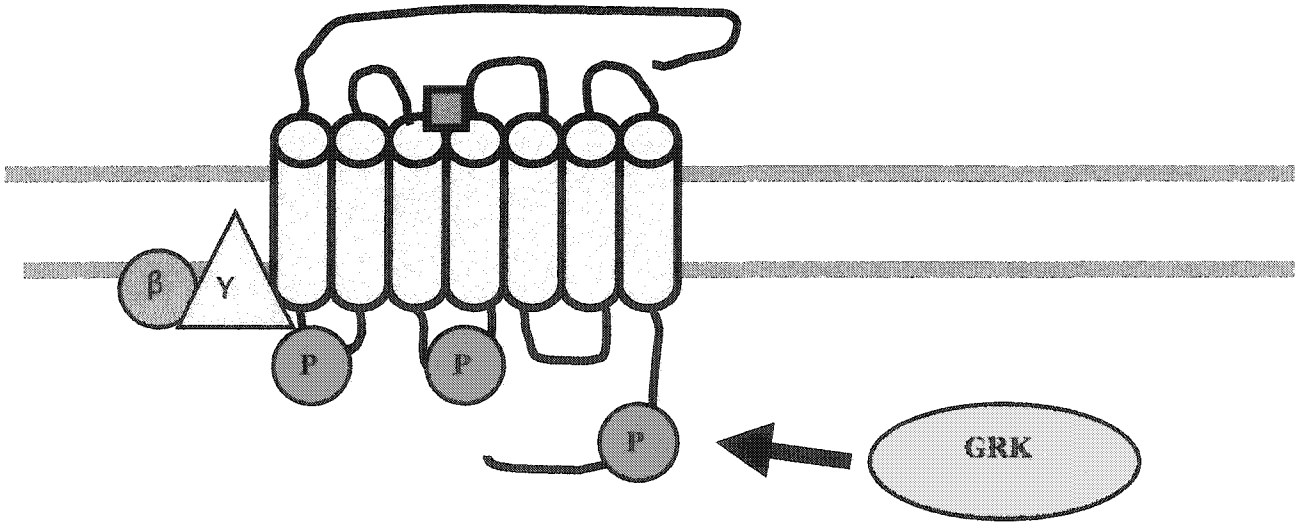
Figure 2 – Events in GPCR activation and signalling. Binding of agonist to the GPCR induces the G-proteins to exchange GDP for GTP, the dissociation of the α subunit of the G-protein from the βγ subunits, and association of the subunits with effector molecules. Agonist binding also promotes phosphorylation of the receptor by GRKs. Phosphorylated GPCRs attract beta-arrestin, which binds to the GPCR, uncouples the receptor from its cognate G-protein, and initiates internalization of the GPCR/ligand complex. Once internalized, the receptor can either be targeted to lysosomes for degradation, dephosphorylated and recycled back to the plasma membrane, or destined to other organelles to continue intracellular signalling.

Figure 2 - Steps in GPCR signaling.

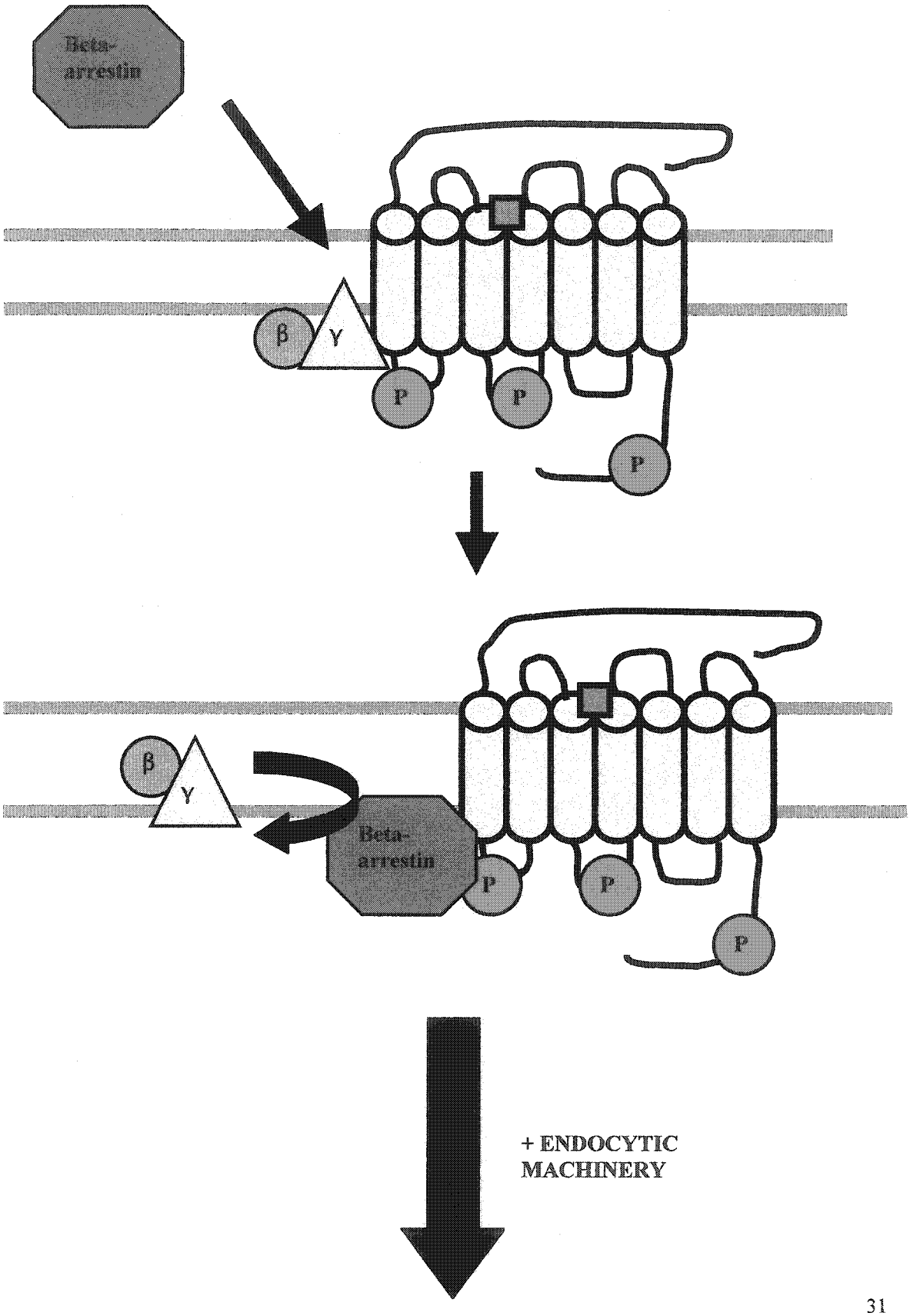
Step 1 – Agonist binding promotes the G-proteins to exchange GTP for GDP, the dissociation of the α subunit from the βγ subunit, and association of the subunits with effector proteins.



Step 2 – Agonist binding also promotes GPCR phosphorylation by GRKs



Step 3 – Phosphorylated GPCRs promote beta-arrestin binding, which mediates receptor-G-protein uncoupling, and internalization of the receptor/ligand complex.



Step 4 – GPCR internalization and intracellular targeting of receptor.

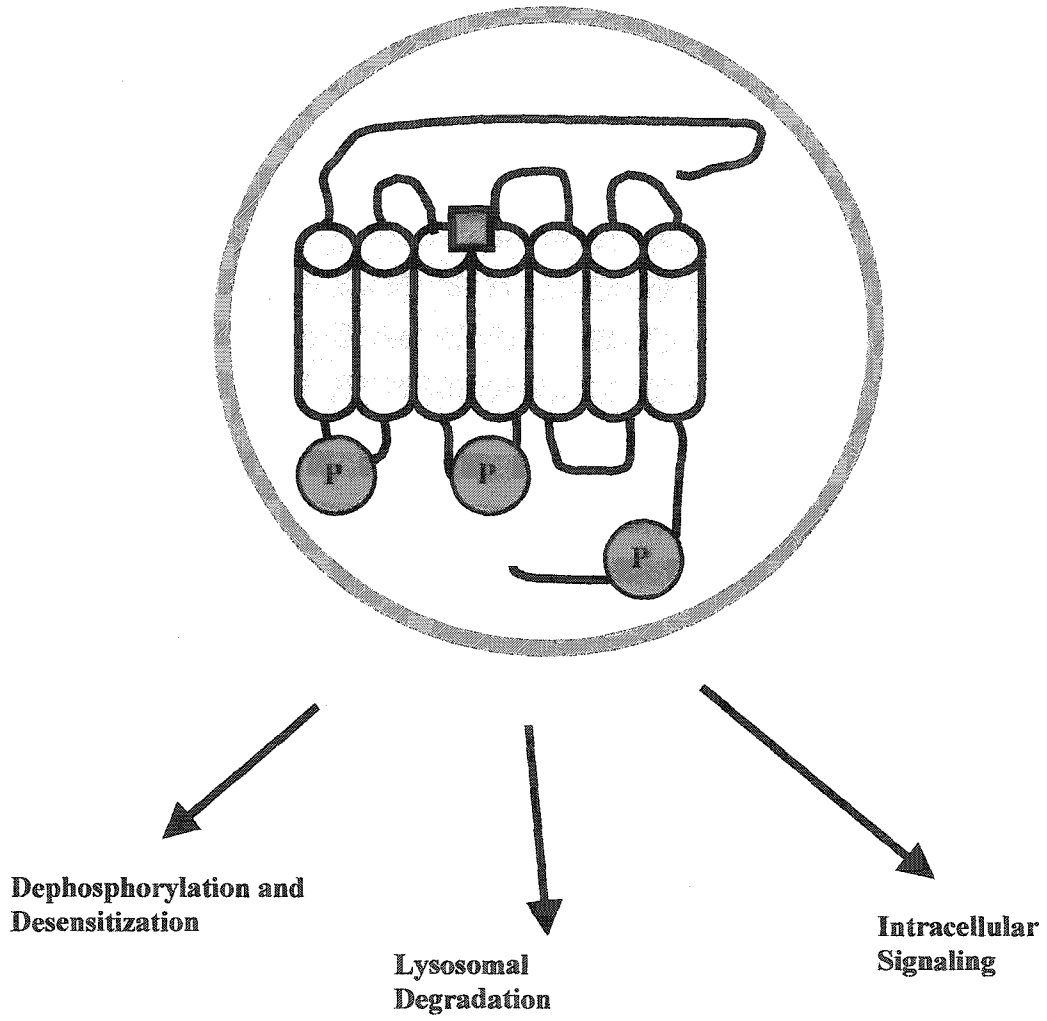
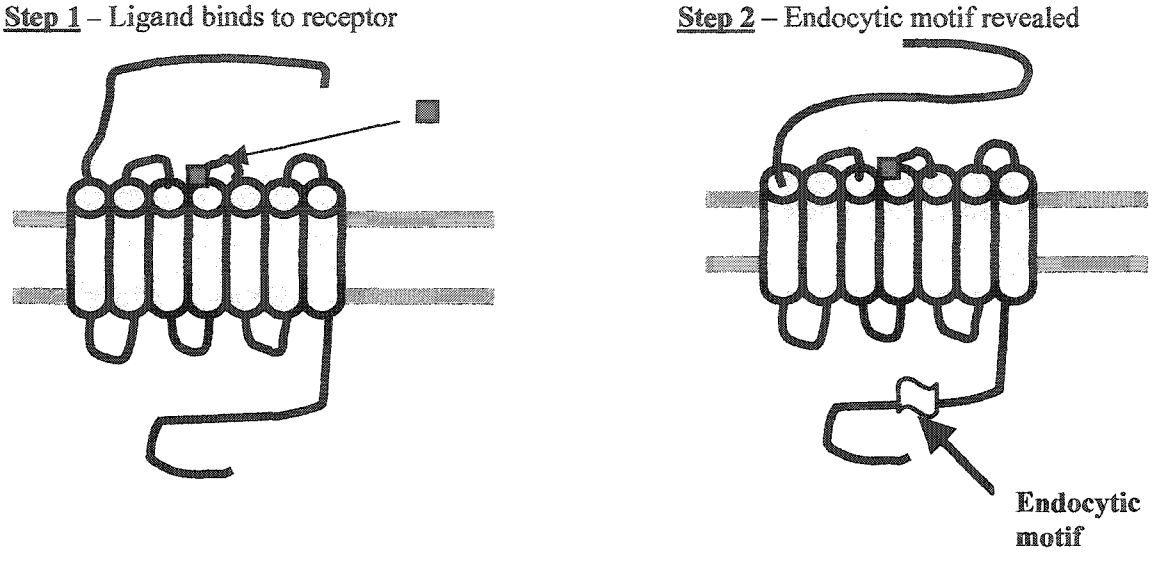


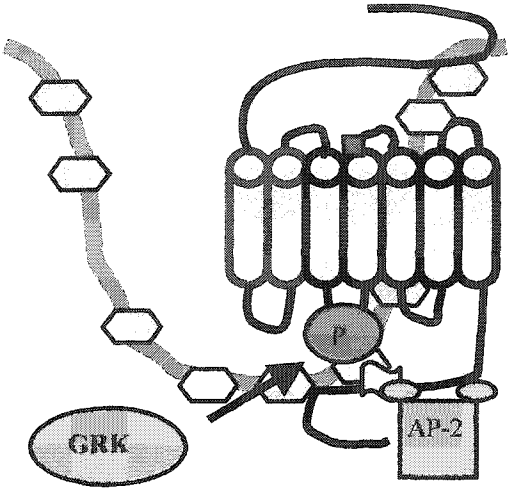
Figure 3 – Steps in GPCR internalization via the clathrin-mediated pathway.

Internalization of GPCRs through the clathrin-mediated pathway begins with the binding of extracellular agonist to the plasma membrane-bound receptor. The binding of the ligand to the GPCR causes the endocytic motif, on the intracellular C-terminus of the receptor, to be revealed. Exposure of the endocytic motif allows the adaptor protein, AP-2, to bind. Once AP-2 has bound to the GPCR/ligand complex, cytosolic clathrin is recruited to the site. Clathrin triskelia self assemble to form a clathrin lattice, which invaginates the plasma membrane at the site of the GPCR/ligand complex. Next, beta-arrestin is recruited to the site of the clathrin-coated pit, where it binds both to the receptor and the clathrin lattice. At the clathrin-coated pit, beta-arrestin both desensitizes the receptor to prevent further agonist stimulation and initiates clathrin-coated vesicle formation. Dynamin is then recruited to the site, probably by binding to either intersectin, amphiphysin, or both. Binding of GTP at the site of the clathrin coated pits initiates dynamin polymerization and the distribution of the polymers around the neck of the coated pit. GTP hydrolysis by dynamin causes the dynamin polymers to constrict the vesicle neck until finally the coated pit is liberated into a clathrin-coated vesicle. Once inside the cell, the clathrin-coated pit is uncoated and the machinery used for internalization is recycled. The uncoated vesicle, containing the ligand and GPCR, then fuses with recycling endosomes, lysosomes, or is transported to other intracellular compartments.

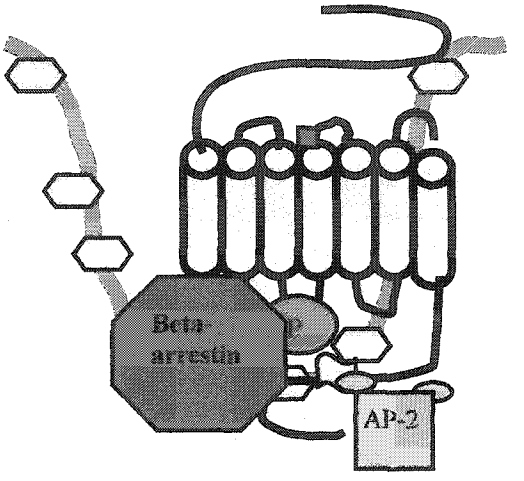
Figure 3 – Steps in internalization of a GPCR via the clathrin-mediated pathway.



Step 5 – Phosphorylation of GPCR by GRKs

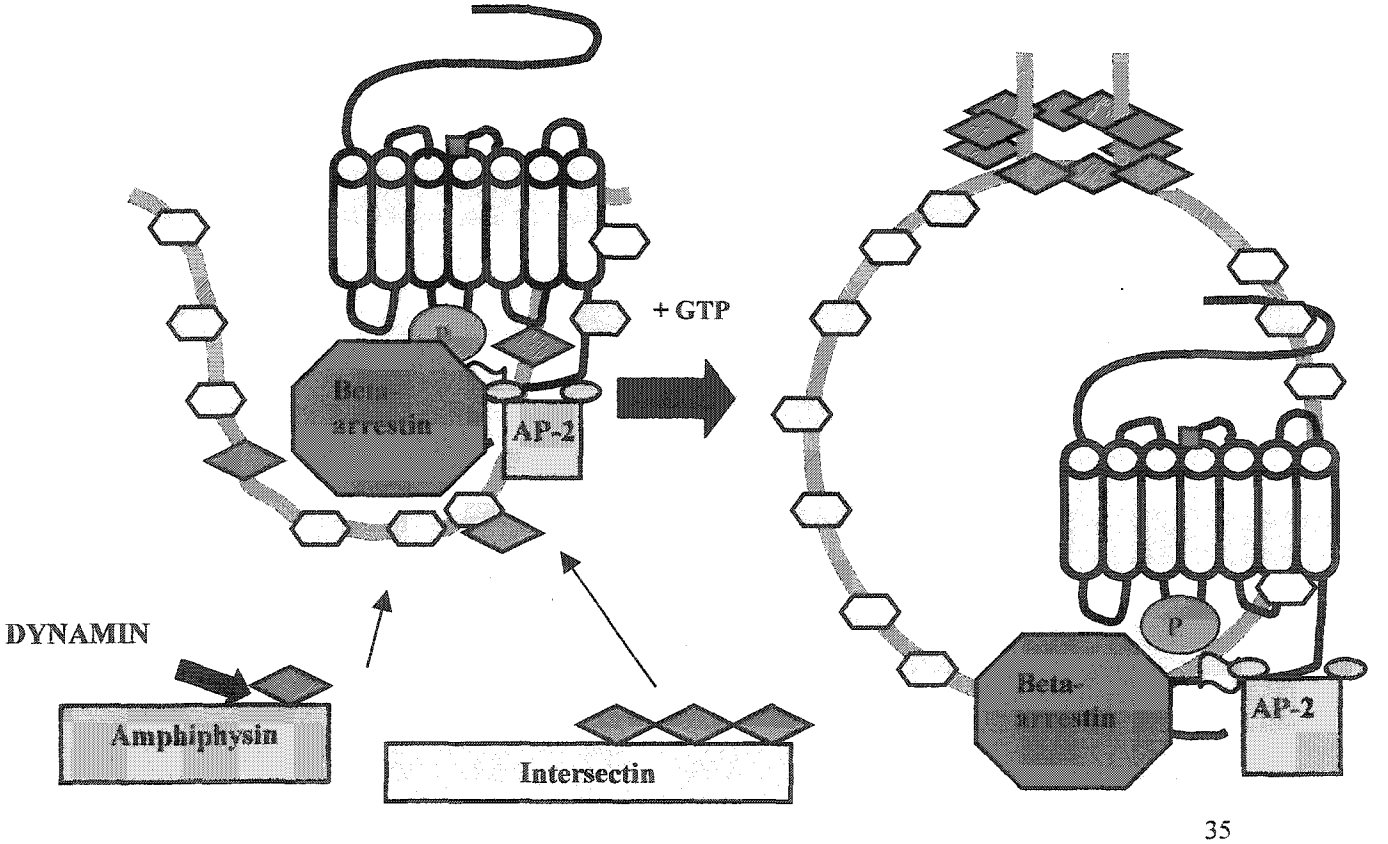


Step 6 – Recruitment of beta-arrestin

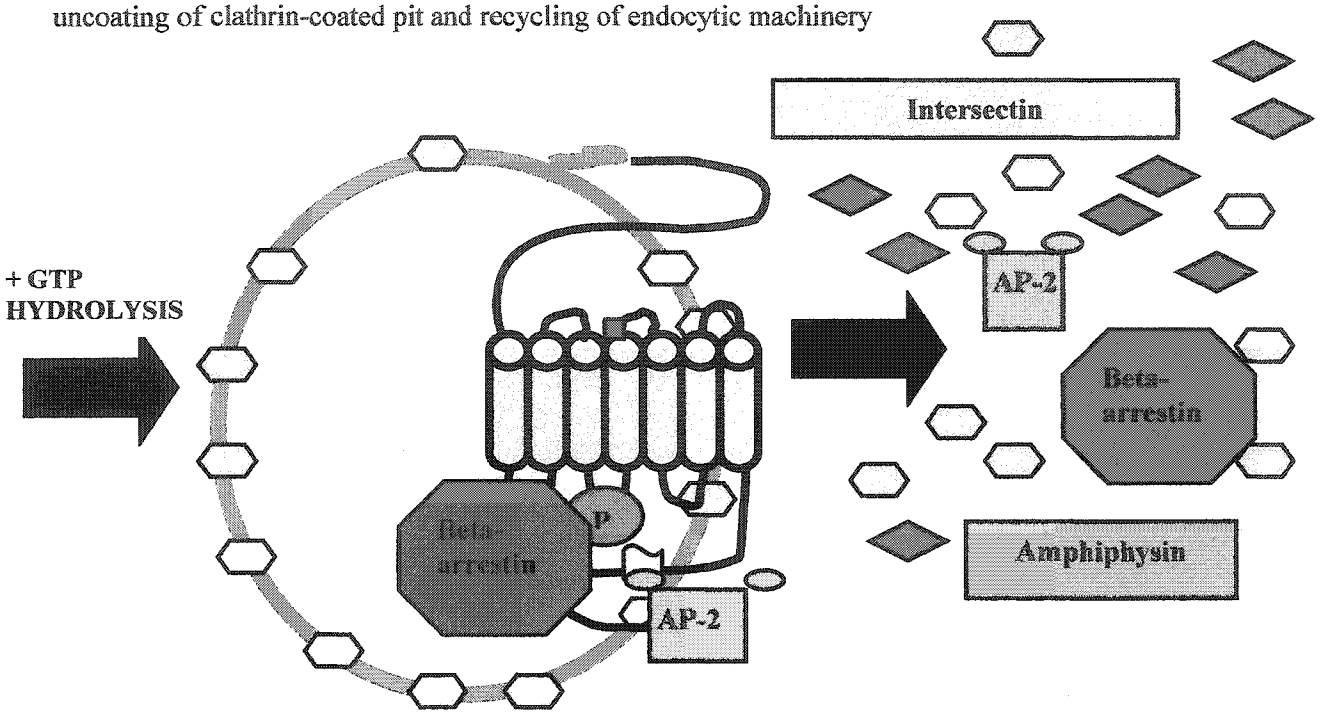


Step 7 – Recruitment of dynamin by intersectin and/or amphiphysin

Step 8 – Redistribution of dynamin to the neck of the clathrin coated pit upon binding GTP



Step 9 - Popping off of coated vesicle upon GTP hydrolysis of dynamin and subsequent uncoating of clathrin-coated pit and recycling of endocytic machinery



Step 10 – Uncoated vesicle fuses with early endosome

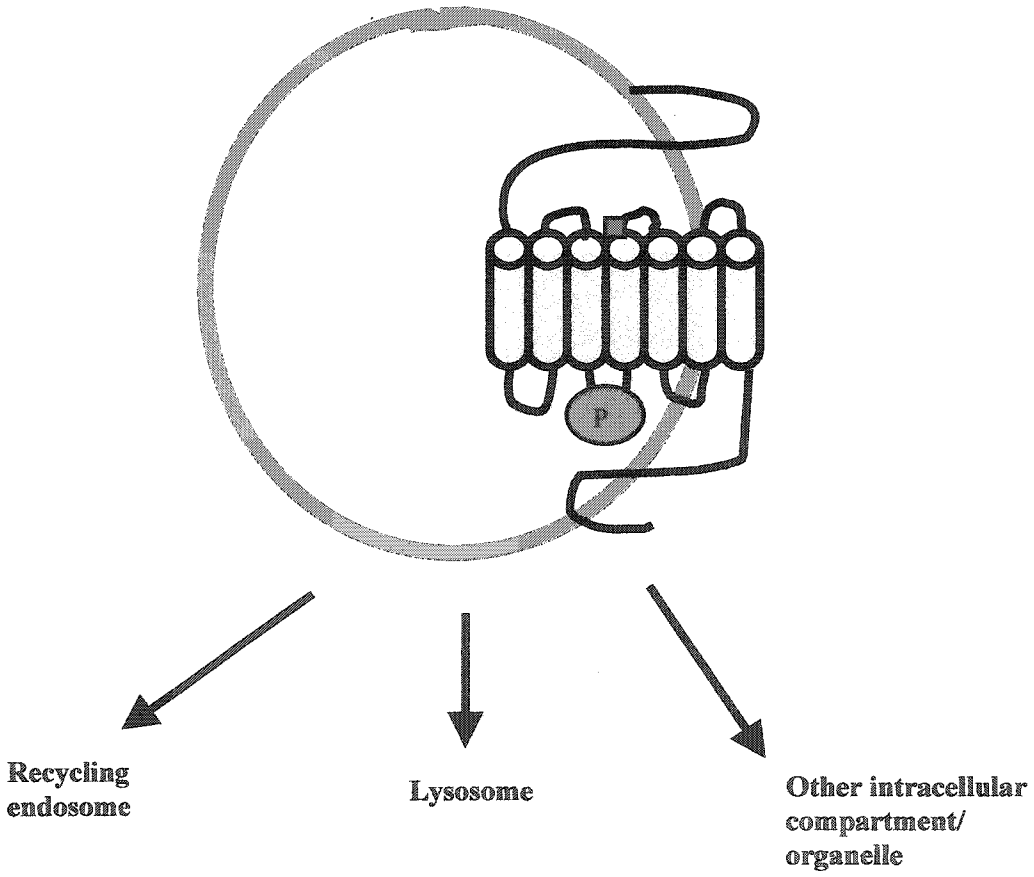
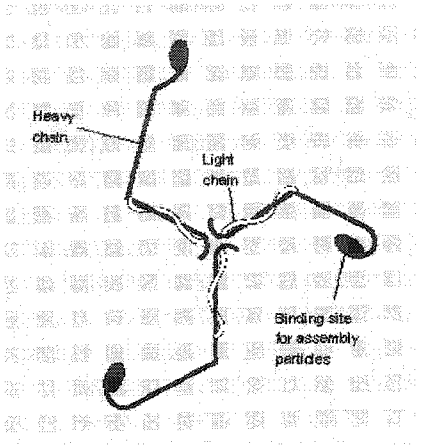
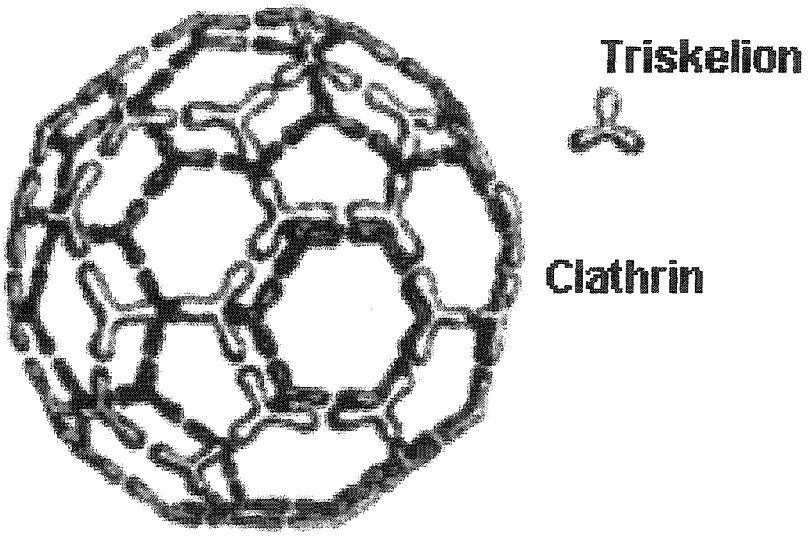


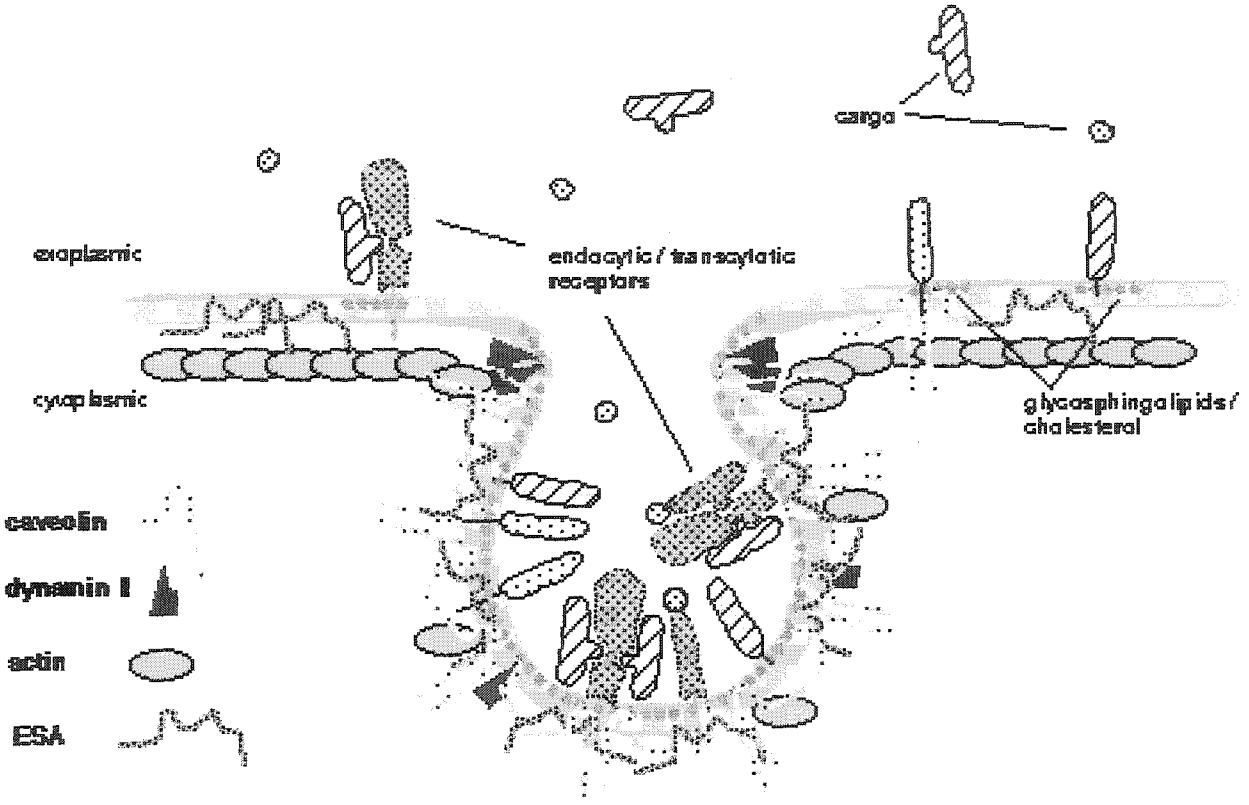
Figure 4 – Diagram of a single clathrin triskelion and an assembled clathrin cage. Shown is a single triskelion, depicting the three heavy chains to which the three light chains are attached (above), as well as the "cage" conformation adopted by free clathrin triskelia (below).





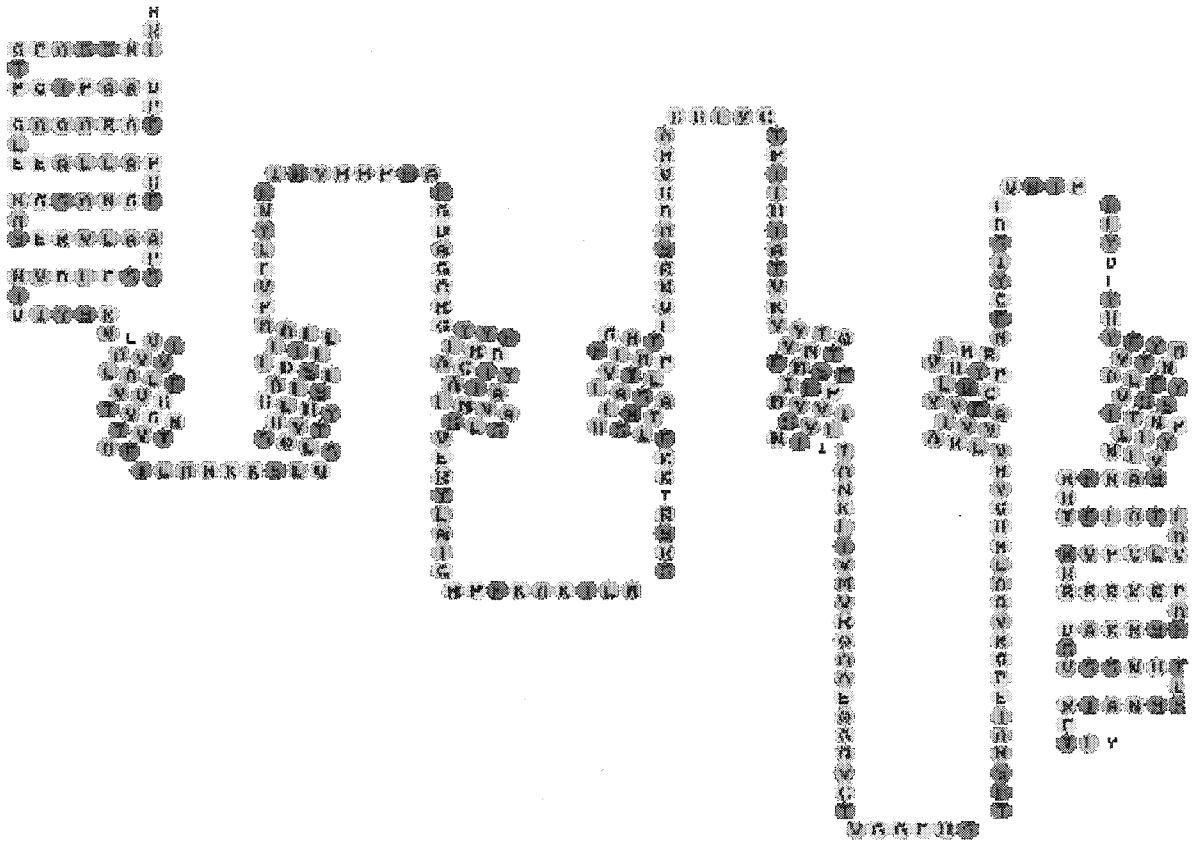
Adapted from: http://www.fortunecity.com/emachines/e11/86/shadow.html

Figure 5 – Structural components of caveolae. Caveolaer pits contain cholesterol and other phospholipids, dynamin, actin, and the 21 kDa protein caveolin.



Adapted from: http://www.biozentrum.unibas.ch/report9899/fiedler.html

Figure 6 – Structure and amino acid sequence of the high affinity neurotensin receptor (NTR1).



Adapted from: http://www.gpcr.org/seq/vis/NTR1_HUMAN/NTR1_HUMAN.html

Figure 7 – Diagram of the signaling cascade initiated by ligand binding to NTR1.

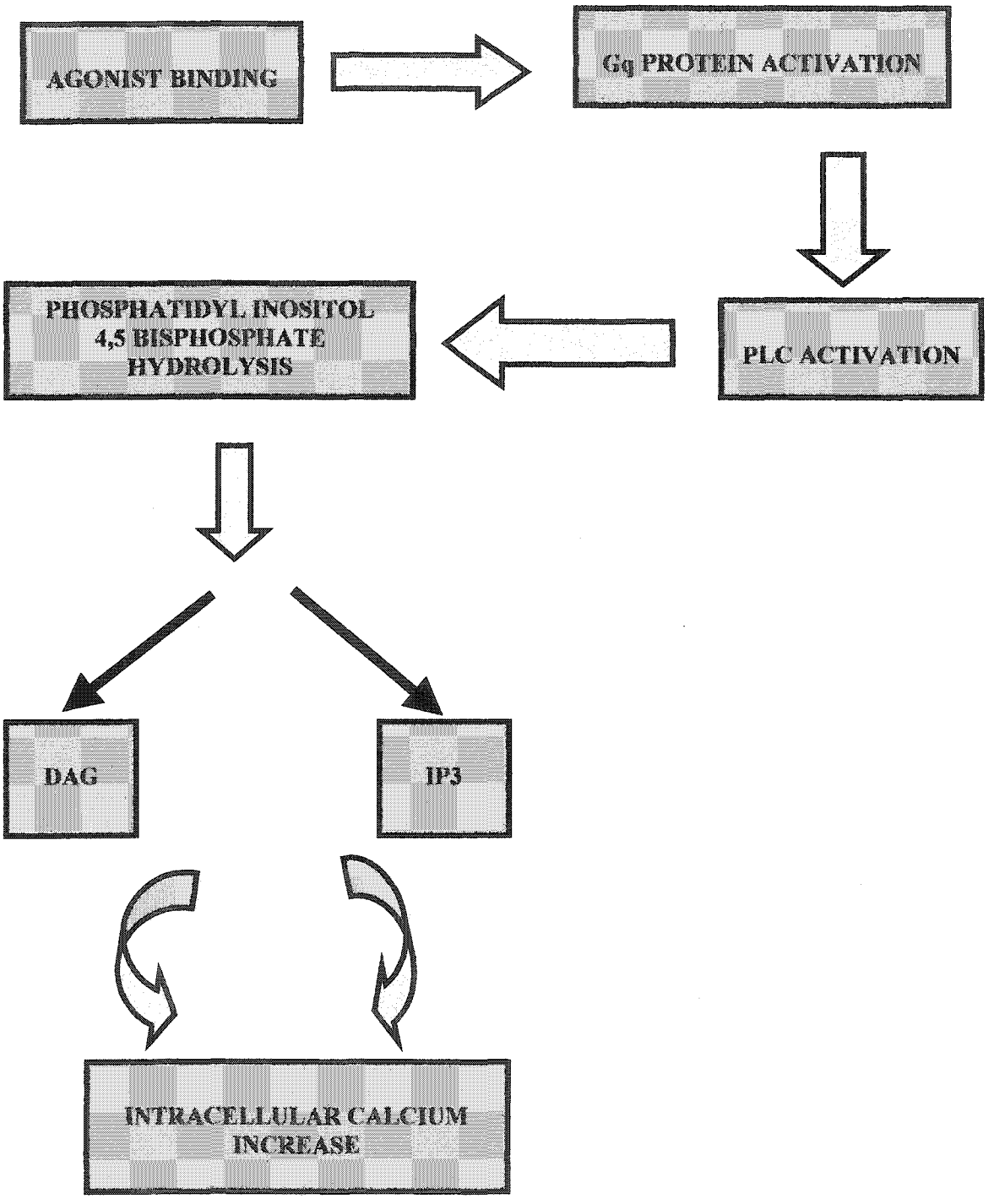


Figure 8 – Diagram of dynamin's domains. Dynamin is composed of an N-terminal GTPase domain, a middle domain, a pleckstrin homology (PH) domain, a GTPase-effector (GED) domain, and a C-terminal proline-rich domain (PRD). "K44A" represents the location at which the K44A dynamin mutant terminates.

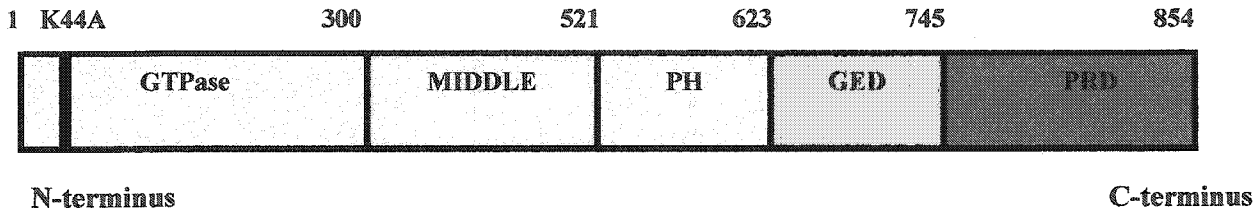
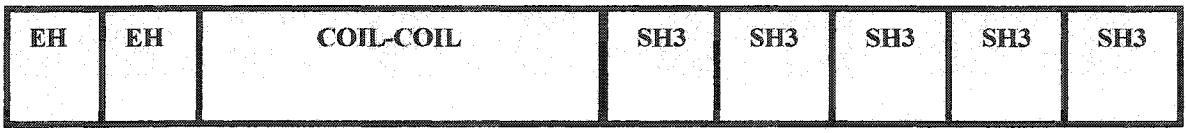


Figure 9 - Diagram of intersectin's domains. Intersectin is composed of two N-terminal EH domains, a middle coil-coil domain, and five C-terminal SH3 domains.



N - terminus **C- terminus**

Figure 10 - Transferrin receptor internalization and recycling pathway.
Extracellular iron-free transferrin binds two iron atoms (Fe^{2+}). Iron-bound transferrin binds to surface transferrin receptors. The transferrin and transferrin receptor enter the cell by endocytosis. Once inside the cell, the endocytic vesicle containing the trasnferrin and transferrin receptor fuses with an endosome. The acidic pH of the endosome destabilizes the iron-transferrin interaction, causing the iron atoms to dissociate from the transferrin molecule. The transferrin remains bound to its receptor, as their interaction is stable at the endosome's pH, and they both recycle back to the plasma membrane. At the pH of the extracellular medium, transferrin dissociates from the transferrin receptor and goes off to once again bind another two atoms of iron. The transferrin receptor remains on the plasma membrane, ready to bind and internalize another molecule of iron-bound transferrin.

Figure 10 - Transferrin receptor internalization and recycling pathway.

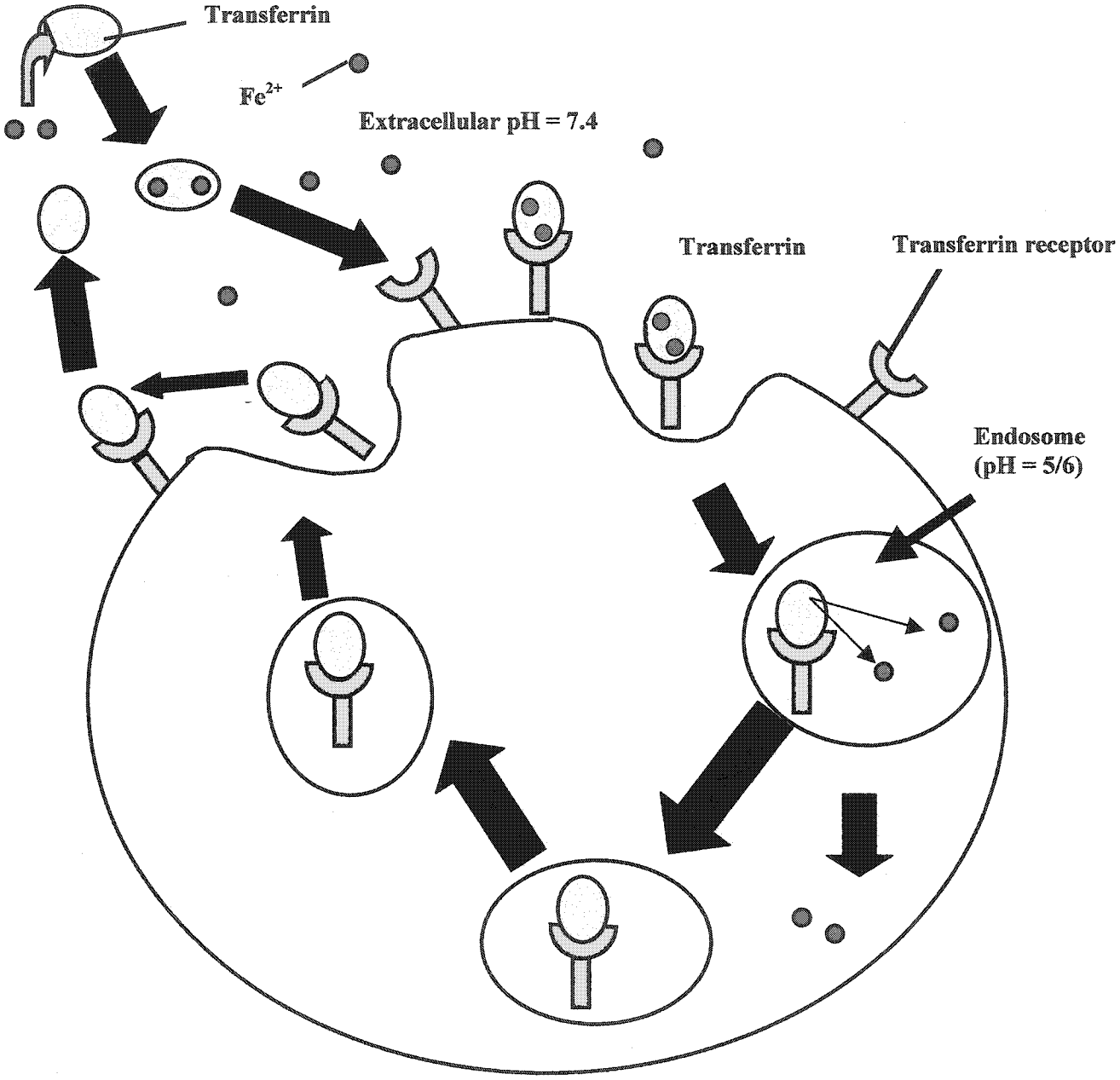


Figure 11 - What proteins are involved in NTR1 internalization?

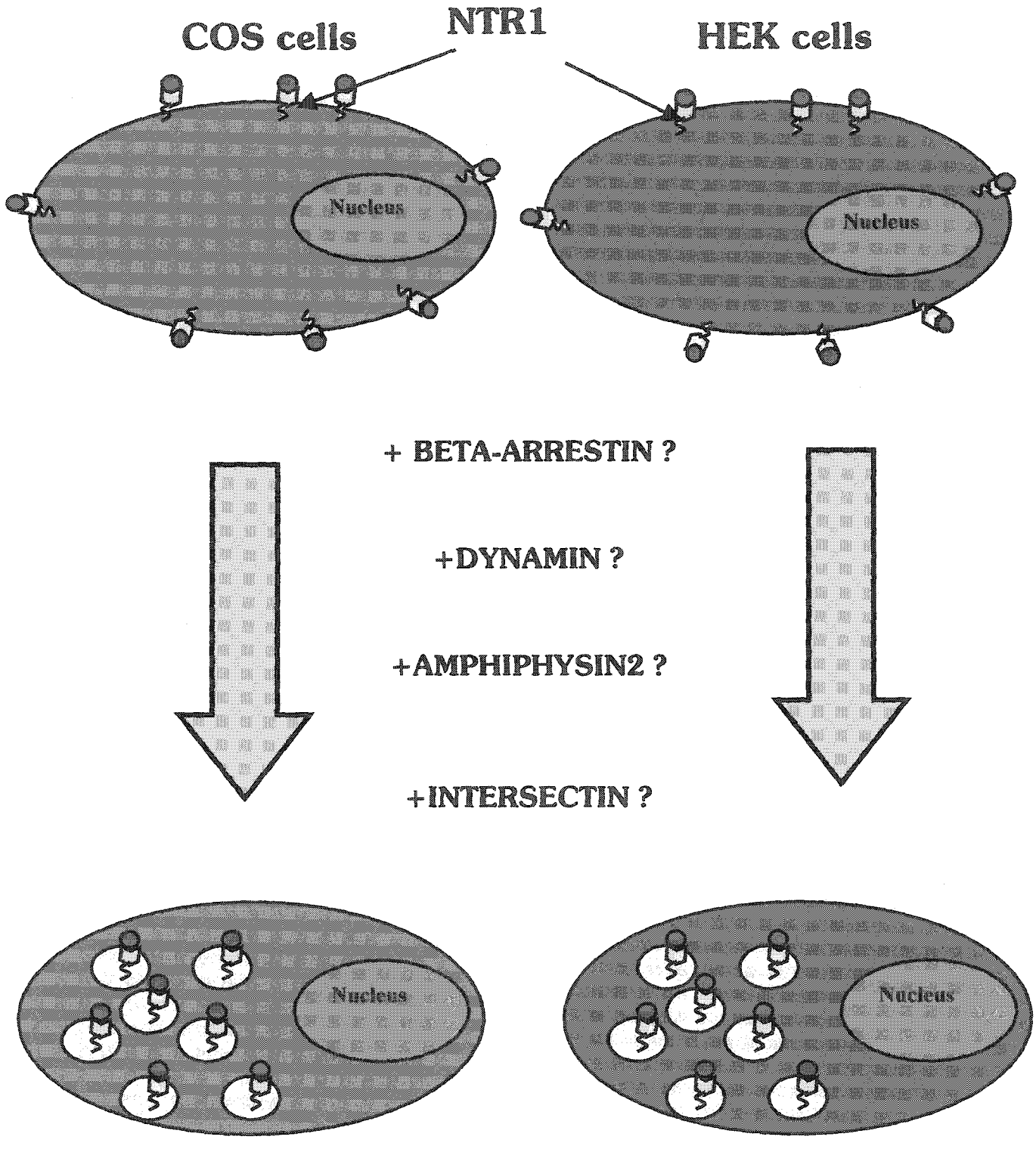


Figure 12 - What proteins are involved in transferrin receptor internalization?

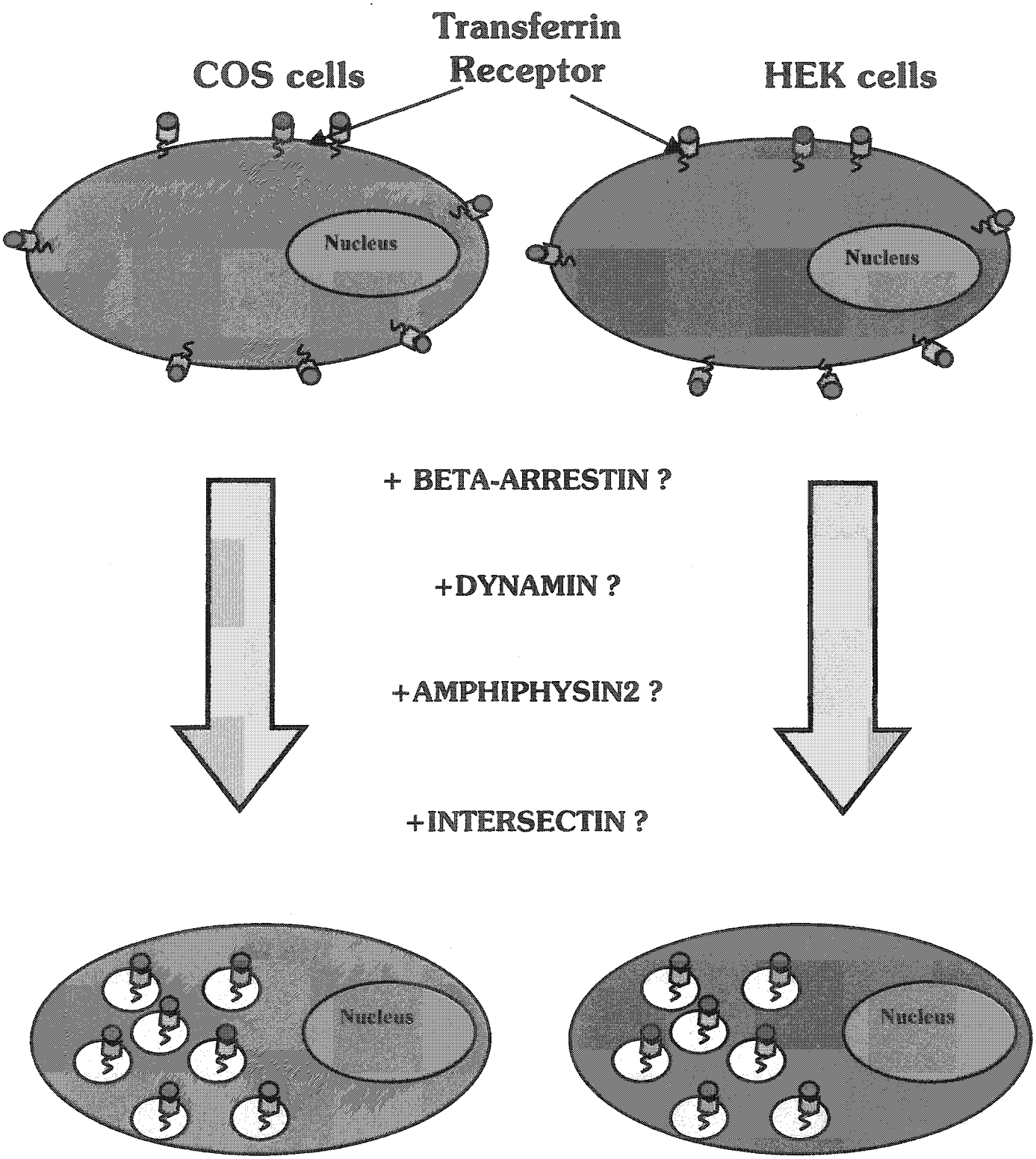


Table 1 – Summary of studies that have looked at the involvement of beta-arrestin in the internalization of GPCRs.

Receptor	Cell type	Beta-arrestin construct used	Effect on receptor internalization	Reference
Beta-adrenergic	COS-7 and HEK 293	V53D	Inhibited	Zhang et al, 1996
Alpha adrenergic subtypes a,b, and c	COS-7	Beta-arrestin1, beta-arrestin2	Enhances	DeGraff et al, 1999
NK-1	KNRK	Beta arrestin mutant (319-418)	Inhibited	McConalogue et al, 1999
Kappa opioid receptor	CHO	Beta arrestin mutant (319-418)	Inhibited	Li et al, 1999
Mu opioid receptor	CHO	V53D	Inhibited	Zhang et al, 1998
Beta-adrenergic	COS, HEK 293, HeLa	Beta arrestin mutant (319-418)	Inhibited	Gangon et al, 1998
LHR	HEK 293	Beta arrestin mutant (319-418)	Inhibited	Lazari et al, 1998
AT1AR	COS-7	V53D	Does not affect	Zhang et al, 1996
Muscarinic m1, m3, m4	HEK 293	V53D	Inhibited	
Muscarinic m1, m3, and m4	HEK 293	Beta-arrestin1	Enhanced	Vogler et al, 1999
Muscarinic m2	HEK 293	V53D	Inhibited	Vogler et al, 1999
Muscarinic m2	HEK 293	Beta-arrestin1	Enhanced	Vogler et al, 1999
CXCR1	HEK 293	Beta-arrestin1	Enhanced	Barlic et al, 1999
CXCR1	RBL-2H3	V53D	Inhibited	Barlic et al, 1999
Secretin	HEK 293	V53D	Not affected	Walker et al, 1999
CXCR4	COS-7 and HEK 293	V53D	Inhibited	Orsini et al, 1999
Endothelin subtypes Eta and Etb	CHO	V53D	Inhibited	Bremnes et al, 2000
Endothelin subtypes Eta and Etb	CHO	Beta-arrestin1	Enhanced	Bremnes et al, 2000
AT1AR	COS, HEK 293, CHO	V53D	Inhibited	Gaborik et al, 2001

Legend:
V53D beta-arrestin binds to clathrin with high affinity but is severly impaired in bidning to phosphorylated GPCRs.
Beta arrestin mutant (319-418) is a construct of beta-arrestin's clathrin-binding domain.

Table 2 – Summary of studies that have looked at dynamin's role in the internalization of GPCRs.

Receptor	Cell type	Dominant Negative Used	Effect on Receptor Internalization	Reference
Beta-adrenergic	COS, HEK	K44A	Inhibits	Zhang et al, 1996
AT1AR	COS, HEK	K44A	Does not affect	Zhang et al, 1996
Beta-adrenergic	COS, HEK, HeLa	K44A	Inhibits	Gagnon et al, 1998
Muscarinic m1, m3, m4	COS , CHO	K44A	Inhibits	Vogler et al, 1998
Muscarinic m2	COS, CHO	K44A	Does not affect	Vogler et al, 1998
Leutinizing hormone	HEK	K44A	Inhibits	Lazari et al, 1998
Dopamine D1	HEK	K44E	Inhibits	Vickery et al, 1999
Dopamine D2	HEK	K44E	Does not affect	Vickery et al, 1999
Bradykinin B2	COS, HEK, CHO	K44A	Inhibits	Pizard et al, 1999
CXCR1	RBL-2H3	K44A	Inhibits	Barlic et al, 1999
Secretin	HEK	K44A	Does not affect	Walker et al, 1999
CXCR4	COS, HEK	K44A	Inhibits	Orsini et al, 1999
Eta and ETb	CHO	K44A	Inhibits	Bremnes et al, 2000
M2	JEG-3	K44A	Does not affect	Schlador et al, 2000
M2	HEK	N272	Inhibits	Werbonat et al, 2000
AT1AR	HEK	N272	Inhibits	Werbonat et al, 2000
AT1AR	COS, HEK, CHO	K44A, K535A	Inhibits	Gaborik et al, 2001
M2	COS, Cardiac myocytes	K44A	Inhibits	Dessy et al, 2000
NK-1	KNRK	K44E	Inhibits	Schmidlin et al, 2001
5-HT2A	HEK	K44A	Inhibits	Bhatnagar et al, 2001

Legend:

N272 – dynamin mutant lacking the N-terminal 272 amino acids (ie deletion of all three dynamin GTP-binding domains)
K535A – dynamin2 mutant impaired in phospholipid binding
K44A – dynamin mutant with decreased GTP binding and hydrolysis activity

Chapter 2 - MATERIALS AND METHODS

2.1 Materials

COS-7 cells, derived from African green monkey kidney epithelial cells, and HEK 293 cells, derived from human embryonic kidney epithelial cells, were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). Dulbecco's Modified Essential Medium (DMEM), Pen/Strep antibiotics, Fetal Bovine Serum (FBS), Trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA-4 Na), and Fungizone were all commercially bought from Gibco BRL, Life Sciences. Ampicillin, tetracycline, and kanamyacin antibiotics, as well as dextran and chloroquine, were purchased from Sigma. MC108 and JM 109 bacterial cells and restriction enzymes (*HindIII, XhoI, XbaI, EcoRI, BstXI, BglII* and *SAlI*) were bought from Promega.

2.2 Cell Culture

COS-7 and HEK 293 cells were grown in 75 cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM) supplemented with 1% Pen/Strep antibiotics, 10% Fetal Bovine Serum (FBS), and 0.5% Fungizone at 37 °C in an atmosphere of 5% CO_2. Cells were passaged every 2-3 days by detaching the cells with 5 ml trypsin for 5 minutes, adding supplemented DMEM to inactivate the trypsin, collecting in a 15 ml test tube, centrifuging the cells into a pellet, re-suspending the pellet in fresh DMEM , and aliquoting into flasks.

2.3 Construction and Expression of constructs

2.3.1 NTR1

The cDNA encoding for the rat NTR1 was a gift from Dr. Shigetada Nakanishi (Institute for Immunology, Faculty of Medicine, Kyoto University, Kyoto, Japan). For eukaryotic expression, the cDNA of NTR1 was inserted into the pcDNA1 vector containing ampicillin and tetracycline resistance genes. MC108 bacterial competent cells (Promega) were transformed with the pcDNA1 vector containing the NTR1 sequence. Positive clones were isolated on 50 ug/ml ampicillin and 10 ug/ml tetracycline-treated agar. The plasmid was amplified using the Quiagen

Quiafilter Midi Kit, and presence of the 1.44 kb insert was confirmed after digestion with *HindIII* and *XbaI*.

2.3.2 Beta-arrestin1 V53D

The cDNA encoding for the beta-arrestin1 V53D mutant was a gift from Dr. Stephen S. Ferguson (The John. P. Robarts Research Institute, London, Ontario, Canada). Briefly, a point mutation in rat beta-arrestin-1 was generated in which the codon GTC, which codes for valine in the 53rd position, was mutated to GAC, which codes for aspartic acid. The resulting mutated cDNA was expressed in pcDNA1 which contained an ampicillin-resistant gene (Ferguson et al, 1996). JM109 bacterial competent cells were transformed with the pcDNA1 plasmid containing the V53D beta-arrestin1 mutant cDNA. Positive clones were identified by growing the cells on agar supplemented with 50 ug/ml ampicillin. The plasmid was amplified using the Quiagen Quiafilter Midi Kit. Presence of the 1.9 kB insert was confirmed following digestion with *HindIII* and *XhoI*.

2.3.3 Dynamin K44A

The cDNA encoding the dynamin mutant K44A was a gift from Dr. Stephen S. Ferguson. The dynamin K44A mutant was originally constructed by van der Bliek and colleagues in 1993. Briefly, site-directed mutagenesis was performed to induce a mutation in the rat dynamin cDNA to mutate codon AAG (lysine) at the 44th position to GCC (alanine). This mutation replaces a critical lysine residue required for nucleotide binding. For eukaryotic expression, the dynamin K44A cDNA was expressed in a pcDNA1 vector which contained an ampicillin-resistant gene. JM109 cells (Promega) were transformed with the plasmid, and positive clones were isolated on agar supplemented with 50 ug/ml ampicillin antibiotic. The plasmid was amplified with the Quiagen Quiafilter Midi kit, and presence of the 860 base pair insert was identified by HindIII and Xho1 digests.

2.3.4 GFP

The pEGFP-C2 plasmid was a gift from Dr. Peter McPherson, Montreal Neurological Institute, Montreal, Canada. JM 109 bacterial cells were transformed with pEGFP-C2 and positive clones were selected on agar plates containing 40 ug/ml kanamycin. The plasmid was

amplified using the Quiagen Quiafilter Midi kit, and presence of the 4.7 kB EGFP insert was confirmed with *EcoRI* and *BstXI* digestion.

2.3.5 GFP – INT

Intersectin was amplified by PCR using the full length cDNA (Yamabhai *et al.*, 1998) with Vent DNA Polymerase (New England Biolabs). The resulting PCR product was digested with Xho1 and EcoR1 and subcloned into pEGFP-C2 (4.7 kB), which adds the N-terminal tag. The resulting plasmid was introduced into JM109 competent bacterial cells, and recombinant plasmids were selected on agar plates containing 40ug/ml kanamycin. Plasmids were amplified using the Quiagen Quiafilter Midi kit, and presence of the 3.6 kB intersectin insert was confirmed with *EcoRI* and *BstXI* digestion. This plasmid was a gift from Dr. Peter McPherson.

2.3.6 GFP – I – SH3

The plasmid containing the tandem SH3 domains of intersectin fused to GFP was a gift from Dr. Peter McPherson. Briefly, a recombinant adenovirus encoding the five tandem SH3 domains of intersectin with an N-terminal GFP tag was produced. First, the appropriate region of intersectin was amplified by PCR using a full length cDNA (Yamabhai *et al.*, 1998) with Vent DNA Polymerase (New England Biolabs). The resulting PCR product was digested with *XhoI* and *EcoRI* and subcloned, in-frame, into the corresponding sites of pEGFP-C2 (Clontech) adding the N-terminal GFP tag. The resulting plasmid was introduced into JM 109 competent bacterial cells, and positive clones were selected on agar plates containing 40ug/ml kanamyacin. Plasmids were amplified using the Quiagen Quiafilter Midi kit, and presence of the insert was confirmed with *EcoRI* and *BstXI* digestion.

2.3.7 GFP – I – EH

A recombinant adenovirus encoding the two tandem EH domains of intersectin with an N-terminal GFP tag was produced. The appropriate region of intersectin was amplified by PCR using a full length cDNA (Yamabhai *et al.*, 1998) with Vent DNA Polymerase (New England

Biolabs). The resulting PCR product was digested with *Xho*I and *Eco*RI and subcloned, in-frame, into the corresponding sites of pEGFP-C2 (Clontech) adding the N-terminal GFP tag. The resulting plasmid was introduced into JM 109 competent bacterial cells, and positive clones were selected on agar plates containing 40ug/ml kanamyacin. Presence of the 800 bp insert was confirmed with by *EcoRI* and *BstXI* digests. This plasmid was provided generously by Dr. Peter McPherson.

2.3.8 GFP – A – SH3

A recombinant adenovirus encoding the SH3 domain of amphiphysin2 with an N-terminal GFP tag was produced with Vent DNA Polymerase (New England Biolabs). The resulting PCR product was digested with *BglII* and *SAlI* and subcloned, in-frame, into the corresponding sites of pEGFP-C2 (Clontech) adding the N-terminal GFP tag. The resulting plasmid was introduced into JM 109 competent bacterial cells, and positive clones were selected on agar plates containing 40ug/ml kanamyacin. Plasmids were amplified using the Quiagen Quiafilter Midi kit, and presence of the 450 bp insert was confirmed with *EcoRI* and *BstXI* digestion. This plasmid was once again a kind gift from Dr. Peter McPherson.

2.4 Origin of Fluorescent Probes

2.4.1 Fluo-NT

Fluo-NT was originally synthesized and purified by Gaudriault and Vincent in 1992. Fluo-NT was obtained by incorporating a fluoresceinyl group on the terminal alpha amine of NT (2-13). This was prepared by reacting the N-hydroxysuccinimide ester of fluorescein with NT at pH 6.5. The product was then purified with reverse-phase HPLC to create N-Bodipy-NT(2-13) or BODIPY-NT (Gaudriault and Vincent, 1992). The fluo-NT (BODIPY-NT) that was used in this study had an excitation of 576 nm and an emission at 589 nm. Dr. Jean-Pierre Vincent (Universite de Nice, Nice, France) kindly provided the fluo-NT that was utilized in the present work.

2.4.2 Fluo-TF

"Fluo-TF" was commercially available and was purchased from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, Pennsylvania, USA). Fluo-TF is a Cy3 derivative of human transferrin.

2.5 Transfection

2.5.1 COS-7 cells

COS-7 cells were transfected via the DEAE-dextran method of transfection. Cells were plated to 60% confluency the day before transfection in 100mm petri dishes. The following is the transfection protocol for one petri dish of COS-7 cells.

Cells are rinsed twice with Transfection Tris-Buffered Saline (TTBS) (25 mM Trisma, 137 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM Na_2HPO_4) before addition of the 1 ml transfection solution. The transfection solution is composed of:

- 4 μg DNA
- 50 μl ddH20
- 850 μl TTBS
- 125 μl of a 4 mg/ml DEAE-dextran solution

Subsequently, at room temperature, the dishes are rotated every 10 minutes for 30 minutes to promote even distribution of the transfection solution. The transfection solution is then removed, and the cells are incubated for 3 hours at 37°C with a solution comprised of:

- 100 μl chloroquine of a 5mg/ml solution
- 10 ml DMEM

Finally, the cells are rinsed twice with TTBS and supplemented with 10 ml fresh DMEM. Forty-eight hours after transfection, the cells are used in the binding and internalization assays.

2.5.2 HEK 293 cells

HEK 293 cells were transfected using the Calcium Phosphate method of transfection. Cells were plated to 60% confluency the day before transfection. The following is the transfection protocol for one petri dish of HEK 293 cells.

One Eppendorf tube contains Solution 1, which is composed of:

- 15 μg DNA
- 62 μl of a sterile, 2M $CaP0_4$ solution
- ddH_2O to bring final volume to 500μl

To a second Eppendorf tube, 500μl of 2 X HBS, (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4), pH 7.1 (Solution II) is added.

Solution I is added drop by drop to Solution II which is continuously being bubbled with air using a Pasteur pipette.

While this final solution is being bubbled, it is then added drop by drop to the HEK 293 cells. Seventeen hours later, the cells are rinsed with TTBS and fresh medium is added (DMEM + 10% FBS). Forty-eight hours later the cells are ready for binding and internalization assays.

Double transfection experiments were performed as above for both cell lines, except 4 μg of each plasmid was used for double transfection of COS-7 cells and 15 μg of each plasmid was used for double transfection of HEK 293 cells (i.e. DNA amount doubled).

Two days after transfection, the cells are trypsinized and plated in 24-well plates containing cover slips coated with Poly-L-Lysine (25μg/ml). Cells are incubated for 1.5 hours at 37°C and 5% CO_2 before the binding experiment.

2.6 Protocols

2.6.1 Fluo-NT binding and internalization assay

Internalization experiments using the NTR1 and fluo-NT have been previously established (Nouel et al, 1997; Vandenbulcke et al, 2000). Cells were equilibrated in Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 25 mM Hepes, pH 7.4), supplemented with 0.09% glucose, 0.2% BSA, and 0.8 mM 1,10-phenanthroline to prevent peptide degradation, for 10 minutes at 37°C. Cells are then incubated in supplemented Earle's buffer containing 20 nM fluo-NT with or without 1 mM of unlabeled NT (to determine non-specific binding) for 45 minutes at 37°C degrees to allow the receptors to internalize the fluo-NT. The cells are then placed on ice and rinsed thoroughly three times with Earle's buffer at 4°C to wash away unbound ligand. Cells are subsequently either acid washed twice for 2 minutes at 4°C , or not, with a solution composed of 0.2 mM acetic acid and 0.5 M NaCl in Earle's buffer, pH 4.0. After the acid wash, cells are rinsed thoroughly again three times to remove the acidic medium. The Poly-lysine-coated cover slips are then mounted on slides with Aquapolymount, with the cells facing upwards, allowed to air dry, and either counted or imaged using a confocal microscope. Figure 13 diagramatically depicts the steps involved in the fluo-NT ligand binding and internalization assay.

2.6.2 Fluo -TF binding and internalization assay

Two days after transfection, cells are trypsinized, plated into $100mm^2$ dishes, and incubated for 1.5 hours at 37°C. They are then rinsed twice with TTBS and incubated with serum-free DMEM for 1 hour at 37°C, 5% CO_2, to avoid binding of transferrin found in the FBS to the transferrin receptors on the cells. Cells are then incubated with Earle's buffer supplemented with 0.09% glucose, 0.2% BSA, and 0.8 mM 1,10-phenanthroline for 10 minutes, and then incubated with a Cy3-tagged transferrin at a concentration of 25µg/ml in supplemented Earle's buffer for 20 minutes. Cells are then immediately placed on ice and washed three times with Earle's buffer at 4°C. No acid wash is performed as fluo-TF does not dissociate from the transferrin receptor between pH 4 – pH 8. Washing the cells in a solution with pH lower than four would compromise the integrity of the cell membrane and is not performed. The Poly-lysine

covered cover slips are then mounted with Aquapolymount with cells facing upwards onto slides, allowed to air dry, and either counted or imaged via confocal microscopy.

2.7 Image Analysis via Confocal Microscopy

Labeled COS-7 and HEK 293 cells were examined under a Leica confocal laser scanning microscope (CLSM) configured with a Leica Diaplan inverted microscope equipped with an argon/krypton laser with an output power of 2-50mV and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica, St.Laurent, Canada). All image-generating and processing operations were performed with the Leica CLSM software package. Images were acquired as single optical sections taken through the middle of the cells and averaged over 32 scans/frame. Bodipy and Cy3 signals were detected by exciting samples with 568 nm, and GFP signals were detected by exciting samples with 488nm. Images were saved on Jazz discs, archived, and mounted using Corel Draw 8.

2.8 Analysis

2.8.1 Fluo-NT binding and internalization experiments

In cells co-transfected with GFP-tagged constructs and NTR1, the total number of GFP positive cells (GFP +) cells in each field were counted, as well as the number of GFP + cells that were co-labeled with fluo-NT. This was performed in cells in which acid wash had been performed (ACID WASH condition), or not (TOTAL condition). Therefore, the "TOTAL condition" reflected the proportion of GFP + cells that were also positively transfected with NTR1, whereas the "ACID WASH condition" reflected the proportion of GFP + cells that had internalized fluo-NT. The results were expressed as the percentage of total GFP + cells that co-labeled with fluo-NT.

The percentage of fluo-NT positive cells in the TOTAL condition represent the binding of fluo-NT to NTR1 that are both on the surface of the cell and those which have internalized inside the cell; thus it represents the total binding of fluo-NT to NTR1. Hypertonic acid wash strips away only surface-bound fluo-NT, so that the percentage of cells positive for fluo-NT binding in the ACID WASH condition represents the proportion of cells that have internalized into the cell.

By 45 minutes, most of the fluo-NT has been shown to internalize (Vandenbulcke et al, 2000), indicating that the percentage of fluo-NT positive cells should not be significantly different between the TOTAL and ACID WASH conditions. If the percentage of fluo-NT positive cells is significantly different when comparing TOTAL and ACID WASH conditions, internalization of fluo-NT (and thus of NTR1) is not efficient, and thus may have been disrupted.

In cells co-transfected with NTR1 and either the beta-arrestin V53D or dynamin K44A plasmid, which were not tagged to GFP, the total number of cells were counted, as well as the number of cells that were labeled for fluo-NT. These results were expressed as the percentage of the total number of cells labeled with fluo-NT.

2.8.2 Fluo -TF binding and internalization experiments

In cells transfected with the GFP-tagged constructs alone, the total number of GFP+ cells in each field was counted as well as the number of cells that were co-labeled for transferrin. The results were expressed as the percentage of total GFP+ cells that co-labeled with transferrin.

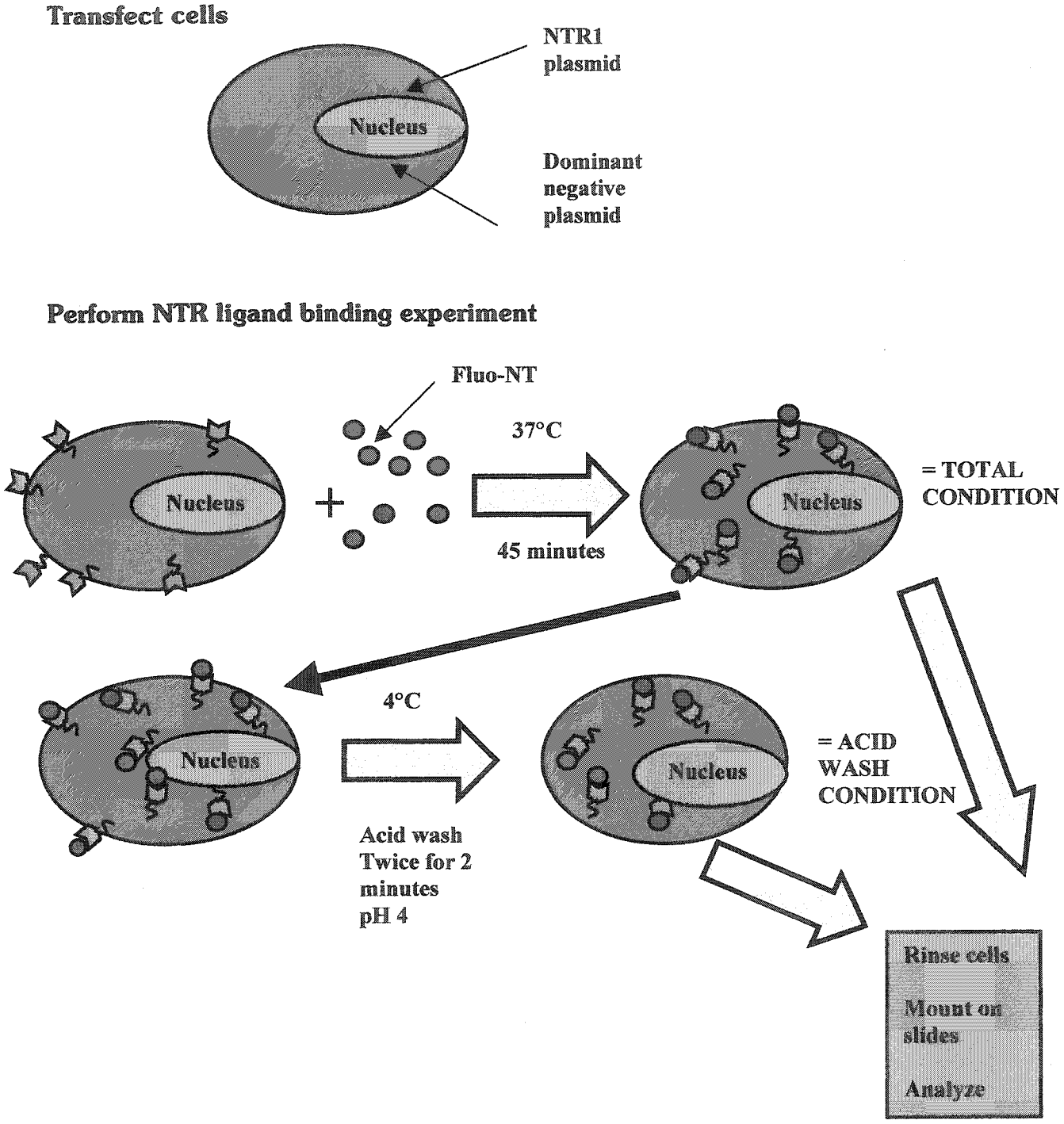
Similarly, in cells transfected with either the beta-arrestin V53D or dynamin K44A plasmid K44A or V53D (not tagged to GFP) alone, the total number of cells was counted, as well as the number of cells that were labeled for fluo-TF. These results were expressed as the percentage of total cells labeled with fluo-TF.

Data were analyzed via the Student's t-test for comparison of independent mean values. Statistically significant results are indicated on the data tables with an asterick, with *** representing $p<0.01$ and ** representing $p<0.05$.

For each condition, the experiment was performed on at least three separate occasions and at least 700 cells were counted per condition (i.e., after 3 separate experiments, the total number of cells counted was at least 700).

Figure 13 – Diagram of NTR1 transfection and ligand binding and internalization assay. COS-7 or HEK 293 cells are transfected either with the NTR1 plasmid alone or co-transfected with the NTR1 plasmid and a dominant negative plasmid. After two days, the cells are subjected to the ligand binding and internalization assay. Cells are incubated with fluo-NT for 45 minutes at 37°C and rinsed, as in the TOTAL condition, or acid washed, and then rinsed, as in the ACID WASH condition. The cover slips containing the cells are then mounted onto slides and analyzed under the confocal microscope.

Figure 13 - Diagram of NTR1 transfection and ligand binding and internalization assay.



Chapter 3 - RESULTS

3.1 Internalization of fluo-NT in non-transfected cells

3.1.1 Internalization of fluo-NT in COS-7 cells

In COS-7 cells transiently transfected with NTR1, a transfection yield of 34 +/- 4% was observed based on the proportion of the total number of cells binding fluorescent neurotensin (fluo-NT). This finding is in line with the results of previous studies reporting transfection yields of 20 - 30% using the same expression vector in the same type of cells (Nouel et al, 1997; Vandenbulcke et al, 2000).

Following 45 minutes of incubation with fluo-NT at 37°C, COS-7 cells singly transfected with NTR1 displayed an accumulation of fluorescent ligand in a single spot located near the nucleus (Figure 14A). As indicated above, the proportion of fluo-NT labelled cells in the total cell population amounted to 34 +/- 4%, and is referred to as the TOTAL condition in Figure 23. Following hypertonic acid wash, labeled cells still exhibited the same pattern of labeling (Figure 14B), indicating that the bulk of the fluo-NT had been sequestered inside the cells. In this condition, shown in Figure 23 as the ACID WASH condition, 30+/- 3% of COS-7 cells were labeled with fluo-NT, demonstrating that virtually all cells expressing the NTR1 receptor internalized the ligand. The specificity of fluo-NT labeling was determined by the lack of specific staining in non-transfected cells and in cells incubated in the presence of 1000-fold excess of non-fluorescent NT (data not shown).

Binding and internalization of fluorescently labelled transferrin (fluo-TF) were observed in COS-7 cells in parallel to fluo-NT binding. As all eukaryotic cells express the transferrin receptor, no transfection of the receptor was necessary and fluo-TF binding was assessed in untransfected cells. As transferrin does not dissociate from its receptor over a wide range of pH (pH 4 – pH 8), hypertonic acid wash, which is carried out at pH 4, was ineffective in this paradigm. However, since all imaging was done on a confocal microscope and all images correspond to single optical sections at a depth of 5 μm from the cell surface, internalized ligand could be identified by virtue of its intracellular location away from the plasma membrane. This criterion was strictly employed to ensure that only cells internalizing fluo-TF were analyzed.

Fluo-TF accumulated in a perinuclear compartment that was somewhat smaller than the one accumulating fluo-NT (compare Figures 14A and 14C). Quantitatively, 65 +/- 4% of the total number of cells were positive for fluo-TF internalization (Figure 24). Although theoretically 100% of untransfected cells should be positive for fluo-TF binding, it is possible that not all the fluo-TF ligand was able to reach the entire cell population on the cover slip. It is also possible that dead or unhealthy cells might not have been able to pick up the ligand. Furthermore, the rigorous standards that were set for identification of a positively fluo-TF labeled cell (as the experiment was not supplemented with the acid wash to confirm intracellular location of ligand) might have passed by some cells that did not bind a critical amount of ligand.

In the presence of 1000-fold excess of non-fluorescent transferrin, cells were completely devoid of labeling, ensuring that the fluo-TF staining was specific to the transferrin receptor (data not shown).

3.1.2 Internalization of fluo-NT in HEK 293 cells

Following 45 minutes incubation at 37°C with HEK 293 cells transfected with NTR1, fluo-NT accumulated over most of the cytoplasm of positively transfected cells (Figure 14D). HEK 293 cells subjected to acid wash treatment after ligand binding lost some of the labeling that was apparent before acid wash (Figure 14D), and only more discrete patches of intracellular staining remained (Figure 14E). Quantitatively, the number of fluo-NT positive cells counted before acid wash (TOTAL condition: 31+/- 7%) was not significantly different from the percentage of fluo-NT positive cells detected after ACID WASH (32 +/- 7%) (n = 5), indicating that labeling after 45 minutes of incubation with fluo-NT was largely intracellular. As deduced from the percentage of fluo-NT positive cells in the TOTAL condition (Figure 25), the transfection efficiency of NTR1 into HEK 293 cells was 32%, in keeping with earlier studies in which NTR1 was transfected into the same cell line (Botto et al, 1998). Specificity of labeling in HEK 293 cells was confirmed through the absence of fluo-NT labeling in non-transfected cells and in cells incubated with 1000 fold excess of non-fluorescent NT (data not shown).

After 20 minutes of incubation of HEK 293 cells with fluo-TF, the fluorescent ligand accumulated in a distinct, intracellular spot (Figure 14F), which was similar in appearance to the fluo-TF accumulation in COS-7 cells (compare Figures 14C and 14F). Quantitatively, 83+/- 3% of HEK 293 cells were positive for fluo-TF labeling (Figure 26), the specificity of which was

established by the absence of labeling upon incubating the cells with a 1000 fold excess of non-fluorescent transferrin (data not shown).

3.2 Beta-arrestin

Beta-arrestin acts as an adaptor between GPCRs and clathrin coated pits as it binds to both GRK-phosphorylated receptors and to AP-2. In order to assess the dependency of NTR1 internalization on beta-arrestin, the beta-arrestin mutant V53D, in which the valine in position 53 is mutated to an alanine, was transfected into COS-7 and HEK 293 cells along with NTR1 and assessed for NTR1 internalization by examining the labeling of fluo-NT. The V53D mutant has a strong affinity for AP2 and clathrin but a reduced affinity for GRK-phosphorylated GPCRs, and has been used to determine the influence of beta-arrestin on the internalization of various GPCRs (see Table 1). It acts as a dominant negative when overexpressed in COS-7 or HEK 293 cells, as most of the beta-arrestin that the cell will use will be of the mutated form. The number of fluo-NT positive cells was counted in a population of cells that were transfected with the V53D mutant and expressed as a percentage of the total number of cells counted per field, for both the TOTAL and ACID WASH conditions.

3.2.1 Effect of overexpressing the beta-arrestin V53D mutant on NTR1 internalization in COS-7 cells

COS-7 cells overexpressing beta-arrestin V53D and the NTR1 receptor exhibited intense cell surface fluo-NT labeling after 45 minutes of incubation with the ligand (Figure 15A). This labeling was not resistant to acid wash (Figure 15B), confirming that it was entirely surface-bound. The quantitative results of this experiment are outlined in Figure 23. In the TOTAL condition, the percentage of COS-7 cells that were fluo-NT positive was 61 +/- 10%, while, in striking contrast, the percentage of fluo-NT positive cells in the ACID WASH condition was significantly lower at 11 +/- 3%. In the presence of beta-arrestin V53D, therefore, the percentage of the cell population that internalized fluo-NT decreased by 82% ($p < 0.01$; $n = 12$). (Note that the transfection efficiency of NTR1 in the presence of beta-arrestin V53D was unusually high).

The labeling of fluo-TF in the presence of V53D took the form of a discrete spot that was clearly intracellular and was no different from the fluo-TF labeling in untransfected COS-7 cells that were incubated with fluo-TF for the same amount of time (data not shown). By contrast to what was observed following incubation with fluo-NT, the percentage of fluo-TF positive COS-7 cells transfected with beta-arrestin V53D (55 +/- 2%) was not statistically significantly different (n = 31) from the percentage of fluo-TF positive cells in untransfected COS-7 cells (65 +/- 4%; Figure 24).

3.2.2 Effect of overexpressing the beta-arrestin V53D mutant on NTR1 internalization in HEK 293 cells

Incubation of fluo-NT in HEK 293 cells transfected with beta-arrestin V53D for 45 minutes at 37°C resulted in a robust cell surface labeling (Figure 15C). This labeling was completely acid-wash sensitive (Figure 15D). Quantitatively, the percentage of fluo-NT positive cells in the TOTAL condition was 39+/- 2%, dropping significantly to 13 +/- 4% under the ACID WASH condition, as depicted in Figure 25. Thus, in the presence of beta-arrestin V53D, the percentage of the cell population that internalized fluo-NT was reduced by 67% ($p < 0.01$; $n = 3$) in HEK 293 cells.

HEK 293 cells expressing the beta-arrestin V53D mutant exhibited a fluo-TF staining pattern similar to that in nontransfected cells. As illustrated in Figure 26, the percentage of fluo-TF positive cells in the presence of the beta-arrestin V53D mutant (77+/- 4%) was not statistically different from the percentage of fluo-TF positive cells seen in non-transfected cells (83 +/- 3%) (n = 10).

3.3 Dynamin

In order to determine if dynamin plays a role in the internalization of NTR1, a mutant form of dynamin, dynamin K44A, was co-transfected with NTR1 in both COS-7 and HEK 293 cells. The number of fluo-NT positive cells was counted and expressed as a percentage of the total number of cells counted per field, for both TOTAL binding and ACID WASH conditions. Dynamin K44A is a dynamin mutant in which the lysine at position 44 is mutated to an alanine, which has the effect of reducing dynamin's affinity for and hydrolysis rate of GTP. Dynamin

K44A had previously been used to determine the influence of dynamin on the internalization of various types of receptors, including several GPCRs (see Table 2).

3.3.1 Effect of overexpressing the K44A mutant dynamin on NTR1 internalization in COS-7 cells

As can be seen in Figure 16A, fluo-NT labeling was intense and confined to the periphery of the cells after 45 minutes of incubation at 37°C in COS-7 cells expressing dynamin K44A. The surface-bound location of fluo-NT was established by subjecting the cells to acid wash, which left no specific fluo-NT staining, but only the faint outlines of the cells' plasma membrane (Figure 16B). In the TOTAL condition (without acid wash), 27 +/- 2% of cells bound fluo-NT, again in keeping with the expected transfection yields in this cell line. When these cells were subjected to acid wash, however, only 6 +/- 1% of the total population of cells were positive for fluo-NT labeling (Figure 23). Thus, the percentage of the cell population that internalized fluo-NT was decreased by 78% ($p < 0.05$; $n = 6$) in the presence of the mutant K44A dynamin in COS-7 cells.

As exhibited in Figure 16C, COS-7 cells expressing the K44A mutant also accumulated fluo-TF at the periphery of the cell. The percentage of fluo-TF positive cells in untransfected COS-7 cells was 65+/- 4%, while in the presence of dynamin K44A, the percentage of fluo-TF labeled cells was decreased markedly to 26 +/- 2% (Figure 24). Therefore, the percentage of the cell population that internalized fluo-TF was reduced by 60% ($p < 0.01$; $n = 34$) in COS-7 cells expressing the dynamin K44A mutant.

3.3.2 Effect of overexpressing the K44A mutant dynamin on NTR1 internalization in HEK 293 cells

Following 45 minutes of fluo-NT incubation at 37°C with HEK 293 cells co-transfected with NTR1 and dynamin K44A, fluo-NT labeling was bright, confined to the periphery of the cell, and was acid-wash sensitive, indicating that the ligand was surface-bound (Figures 16D and 16E). Quantitatively, the percentage of total HEK 293 cells that were positive for fluo-NT was 39 +/-7% before acid wash (TOTAL condition), while the percentage of NT positive cells decreased to 12% +/- 4% following ACID WASH (Figure 25). The difference in the percentage

of fluo-NT binding in the TOTAL and ACID WASH conditions was statistically significant, such that the percentage of the cell population that internalized fluo-NT decreased by 70% ($p < 0.05$; $n = 4$) in HEK 293 cells in which the mutant dynamin K44A was expressed.

Fluo-TF labeling of the majority of HEK 293 cells transfected with dynamin K44A was detected along the perimeter of the cell, and hence were not counted as being positive for fluo-TF internalization (Figure 16F). In untransfected cells, the percentage of HEK 293 cells that bound fluo-TF was 83+/- 3%, and in the presence of dynamin K44A, the percentage of HEK 293 cells that exhibited intracellular fluo-TF labeling decreased by about half to 44 +/- 2% (Figure 26). The quantitative data illustrate that the percentage of cells that internalized fluo-TF was reduced by 47% ($p < 0.01$; $n = 8$) in HEK 293 cells expressing the mutant dynamin K44A.

3.4 GFP

As the following sets of experiments used various proteins tagged to GFP, the effect of GFP alone on fluo-NT and fluo-TF internalization was first investigated. To assess the effect of GFP on fluo-NT internalization in COS-7 cells, GFP was co-transfected into COS-7 cells along with NTR1 and the transfected cells were monitored for fluo-NT internalization. Internalization of NTR1 was induced by incubating the cells at 37°C with fluo-NT for 45 minutes. Subsequently, they were subjected (ACID WASH condition) or not (TOTAL condition) to a hypertonic acid wash (pH 4) to strip off plasma membrane-bound ligand. For each field of cells, the number of cells that were co-labeled for both GFP and fluo-NT were counted. Results were expressed as the percentage of GFP labeled cells that were co-labeled with fluo-NT.

3.4.1 Effect of overexpressing GFP on NTR1 internalization in COS-7 cells

As shown in Figure 17A, GFP staining was present throughout the cell, and the same cell stained for fluo-NT showed a clear accumulation of the ligand in a single intracellular spot near the plasma membrane, similar to the fluo-NT staining seen in COS-7 cells transfected with NTR1 alone (compare Figures 14B and 17A'). The staining pattern remained the same with and without acid wash (data not shown). The number of GFP positive cells were counted, as well as the number of those GFP positive cells that were co-labeled with fluo-NT. Results were expressed as

the percentage of total GFP positive cells that co-labeled with fluo-NT, for both the TOTAL and ACID WASH conditions (Figure 27). In the TOTAL condition, the percentage of total GFP positive COS-7 cells that co-labeled with fluo-NT was 18 +/- 6%, while in the ACID WASH condition, the percentage of total GFP positive COS-7 cells co-labeled with fluo-NT was 12 +/- 6%, a difference that was not statistically significant (n = 16).

In COS-7 cells singly transfected with GFP, the percentage of GFP positive cells that were co-labeled with fluo-TF was 57+/- 3% (Figure 28). Again, whereas COS-7 cells transfected with GFP exhibited a homogeneous distribution, fluo-TF accumulation was intracellular and punctate, as seen in non-transfected COS-7 cells (data not shown).

3.4.2 Effect of overexpressing GFP on NTR1 internalization in HEK 293 cells

As in COS-7 cells co-transfected with GFP and NTR1, GFP staining in HEK 293 cells coexpressing GFP and NTR1 was found throughout the cytoplasm of the cells (Figure 17B) whereas fluo-NT staining was confined to punctate intracellular compartments. This fluo-NT staining was intracellular as determined by its persistance subsequent to acid wash (Figure 17B'). As represented in Figure 29, in the TOTAL condition, 36 +/- 2% of GFP positive cells co-labeled with fluo-NT, and in the ACID WASH condition, this value was 37 +/- 1%, a difference that was not statistically significant (n = 6).

Staining for fluo-TF accumulated in a distinct, bright spot towards the the middle of the cell in the presence of GFP as depicted in Figures 17C and 17C'. As illustrated in Figure 30, 80 +/- 2% of total GFP positive cells were co-labeled with fluo-TF.

3.5 Intersectin

To determine whether intersectin was involved in the receptor-mediated endocytosis of NTR1, the receptor was co-transfected with a GFP-tagged intersectin construct (GFP-INT) in both COS-7 and HEK 293 cells. Internalization of NTR1 was induced by incubating the cells at 37°C with fluo-NT for 45 minutes. Subsequently, the cells were subjected (ACID WASH condition) or not (TOTAL condition) to a hypertonic acid wash (pH 4) to strip off plasma

membrane-bound ligand. For each field of cells, the number of cells that were co-labeled for both GFP- INT and fluo-NT were counted. Results were expressed as the percentage of GFP- INT cells that were co-labeled with fluo-NT.

3.5.1 Effect of overexpressing GFP-INT on NTR1 internalization in COS-7 cells

GFP-INT staining in COS-7 cells co-transfected with GFP – INT and NTR1 was punctate and distributed throughout the entire cell (Figure 18A). This GFP-INT staining pattern remained similar regardless whether the cells were stimulated with fluo-NT or not. The GFP-INT staining also remained unchanged regardless of the time period with which the cells were stimulated with fluo-NT (i.e. from 0 – 45 minutes). Fluo-NT staining in the same cell was surface-bound (Figure 18A'), as it was not resistant to acid wash (Figure 18B'). Quantitatively, 29+/- 4% of GFP – INT positive cells co-labeled with fluo-NT in the TOTAL condition, while only 12 +/- 2% of GFP - INT positive cells were co-labeled with fluo-NT after ACID WASH (Figure 27). The difference in the percentage of GFP - INT cells co-labeled with fluo-NT between TOTAL and ACID wash conditions was statistically significant and indicated that the percentage of cells that the percentage of cells that internalized fluo-NT was reduced by 58% ($p < 0.01$; $n = 12$) in COS-7 cells expressing GFP-INT.

Similarly, fluo-TF exhibited a peripheral staining pattern in COS-7 cells transfected with GFP-INT (Figures 18C and 18C'). Only 30 +/- 4% of total GFP-INT positive cells were co-labeled with fluo-TF (Figure 28). This percentage was significantly different from that of GFP positive cells co-labeled with fluo-TF in cells transfected with GFP alone (57 +/- 3%) (Figure 28). Thus, the expression of GFP-INT in COS-7 cells decreased the percentage of cells that internalized fluo-TF by 47% ($p < 0.01$; $n = 19$).

3.5.2 Effect of GFP-INT on NTR1 internalization in HEK 293 cells

HEK 293 cells co-transfected with GFP-INT and NTR1 showed the same punctate distribution of GFP-INT throughout the cell as seen in dually transfected COS-7 cells, although each puncta appeared to be larger than those seen in COS-7 cells (compare Figure 18A with Figure 18D). Cells expressing GFP – INT internalized fluo-NT, as evidenced by the ability of the labeling to withstand acid wash (Figure 18D'). Quantitatively, as depicted in Figure 29, the percentage of GFP-INT positive cells co-labeled with fluo-NT before acid wash (TOTAL condition: 57 +/- 7%) was not significantly different from the percentage of GFP-INT cells co-labeled with fluo-NT after ACID WASH (51 +/- 9%).

For transferrin, in contrast, although the GFP-INT labeling remained punctate and visible throughout the cell, fluo-TF staining was detected only on the plasma membrane in cells transfected with GFP-INT, and these cells were not counted as being positive for fluo-TF internalization (data not shown). The quantitative results are coherent with these qualitative observations. As represented in Figure 30, in HEK 293 cells transfected with GFP – INT, the percentage of GFP-INT positive cells co-labeled with fluo-TF was 22 +/- 2%, while in HEK 293 cells transfected with GFP alone, the percentage of GFP positive cells co-labeled with fluo-TF was 80 +/- 2%, indicating that the percentage of cells that internalized fluo-TF was reduced by 72% ($p < 0.01$; $n = 28$).

These results indicate that intersectin is involved in transferrin receptor, but not NTR1 internalization in HEK 293 cells.

3.5.1.1 Effect of overexpressing the GFP-tagged SH3 domains of intersectin on NTR1 internalization in COS-7 cells

To determine whether or not the SH3 domains of intersectin were involved in the internalization of NTR1, COS-7 cells co-expressing NTR1 and a GFP-tagged SH3 domain construct of intersectin (GFP-I-SH3) were incubated with fluo-NT for 45 minutes at 37°C and monitored for ligand internalization. The distribution of GFP- I - SH3 was not punctate, as for its full length counterpart, but rather homogeneous throughout the entire cell (Figures 19A, B). Figure 19A' depicts the labeling of fluo-NT in the presence of GFP- I - SH3, which is extremely

bright and entirely plasma membrane-associated, given its disappearance after cells were subjected to acid wash (Figure 19B'). In the TOTAL condition, the percentage of GFP- I - SH3 positive cells that co-labeled with fluo-NT was 31% +/- 3%, as seen in Figure 27, while in the ACID WASH condition, this value was 11% +/- 2%, and was statistically significantly different from the TOTAL condition. Thus, the percentage of the cell population that internalized fluo-NT decreased by 65% ($p < 0.01$; $n = 18$) in COS-7 cells expressing intersectin's SH3 domains.

Similarly, fluo-TF exhibited a peripheral staining in the presence of GFP-I-SH3 in COS-7 cells that were transfected with the GFP-tagged SH3 domain of intersectin, and thus were not considered positive for fluo-TF internalization. The SH3 domains of intersectin were similarly distributed homogeneously throughout the cells (data not shown). In this case, the percentage of GFP-I-SH3 positive cells staining positive for fluo-TF was 43 +- 4% (Figure 28). This value was significantly different from the percentage of GFP positive cells co-labeled with fluo-NT in COS-7 cells transfected with GFP alone (Figure 28), and the percentage of cells that internalized fluo-TF was reduced by 26% ($p < 0.05$; $n = 20$) in cells co-expressing GFP-I-SH3, a modest, yet statistically significant value.

3.5.2.1 Effect of overexpressing the GFP-tagged SH3 domains of intersectin on NTR1 internalization in HEK 293 cells

The labeling pattern of GFP- I - SH3 in dually transfected HEK 293 cells was similar to that seen in COS-7 cells transfected with the same plasmids (Figure 19C). However, in HEK 293 cells, fluo-NT staining displayed a compact intracellular accumulation different from the peripheral labeling seen in COS-7 cells after 45 minutes (compare Figures 19A' and 19C'). This fluo-NT labelling was indeed intracellular as confirmed by its resistance to treatment with hypertonic acid buffer (Figure 19D and 19D'). The percentage of GFP-I-SH3 positive cells co-labeled with fluo-NT in the TOTAL condition was 42% +/- 6%, while in the ACID WASH condition, this value was 39% +/- 4%, a difference which was not statistically significant (Figure 29).

Fluo-TF staining remained entirely surface-bound after 45 minutes incubation with fluo-TF of cells overexpressing GFP-I-SH3 (Figure 20A and 20A'), and were not counted as positive for fluo-TF internalization. Quantatatively, as per Figure 30, only 28 +/-20% of GFP-I-SH3

positive cells co-labeled for fluo-TF, whereas 80+/- 13% of cells transfected with GFP alone co-labeled for fluo-TF. Thus, the percentage of HEK 293 cells that internalized fluo-TF was decreased by 66% ($p < 0.05$; $n = 27$) in the presence of GFP-I-SH3 .

These results indicate that intersectin's SH3 domains are involved in transferrin receptor, but not NTR1 internalization in HEK 293 cells.

3.5.1.2 Effect of overexpressing the GFP-tagged EH domains of intersectin on NTR1 internalization in COS-7 cells

As GFP-I-SH3, the GFP-tagged EH domains of intersectin (GFP-I- EH) staining was distributed homogeneously throughout the dually transfected (GFP-I-EH/NTR1) COS-7 cells (Figure 21A). In these cells, fluo-NT gathered in a distinct perinuclear region that was acid-wash resistant (Figure 21A'). As illustrated in Figure 27, in the TOTAL condition (i.e. without acid wash), 21% +/- 4% of total GFP-I-EH positive cells co-labeled with fluo-NT, while after acid wash, 17+/- 5% of total GFP-I-EH positive cells were co-labeled with fluo-NT, a value not statistically significantly different from the TOTAL condition.

Similarly, fluo-TF accumulated in a single spot that was clearly intracellular in cells expressing the GFP-I-EH construct (data not shown). Quantitatively, there was no significant difference between the percentage of GFP-I-EH positive cells co-labeled with fluo-TF (68 +/- 4%) and the percentage of GFP positive cells co-labeled with fluo-TF (57 +/-3%) (Figure 28).

3.5.2.2 Effect of overexpressing the GFP-tagged EH domains of intersectin on NTR1 internalization in HEK 293 cells

HEK 293 cells expressing GFP-I-EH (Figure 21B) were not decreased from accumulating fluo-NT intracellularly, as shown by both the pattern of fluo-NT labeling and by the fact that this labeling was acid wash resistant (Figure 21B'). Quantitatively, as shown in Figure 29, 36+/-3% of total GFP-I-EH expressing HEK 293 cells were co-labeled with fluo-NT prior to acid wash, whereas 37 +/- 1% of GFP-I-EH cells were co-labeled with fluo-NT in the ACID WASH condition, a difference that was not statistically significant.

Fluo-TF, on the other hand, displayed surface labeling in HEK 293 cells expressing GFP-I-EH (Figure 21C and 21C'), and only 40 +/- 4% of cells expressing GFP-I-EH co-labeled with fluo-TF that was clearly internalized, compared with 80 +/- 2%, which is the percentage of GFP expressing HEK 293 cells that co-labeled with fluo-TF (Figure 30). Thus, the percentage of cells that internalized fluo-TF was reduced by 50% ($p < 0.01$; $n = 18$) in HEK 293 cells expressing GFP-I-EH.

3.6 Amphiphysin

To test whether amphiphysin2 was required for the internalization of NTR1, the SH3 domain of amphiphysin tagged with GFP (GFP-A-SH3) was co-transfected into COS-7 and HEK 293 cells along with NTR1 and assessed for fluo-NT internalization under TOTAL and ACID WASH conditions.

3.6.1 Effect of overexpressing the GFP-tagged SH3 domain of amphiphysin2 on NTR1 internalization in COS-7 cells

In COS-7 cells dually transfected with GFP-tagged SH3 amphiphysin2 (GFP-A-SH3) and NTR1, GFP-A-SH3 was found throughout the entire cell (Figure 22A). By constrast, within the same cells, fluo-NT labeling formed punctate accumulations within the juxta-nuclear area (Figure 22A'). This labeling was resistant to acid wash, confirming that it was intracellular. Quantitatively, the percentage of GFP-A-SH3 co-labeled with fluo-NT following 45 minutes of incubation with the ligand and before acid wash (TOTAL condition), was 16% +/- 2% (Figure 27). After acid wash, the percentage of GFP-A-SH3 cells co-labeled with fluo-NT was not statistically significantly different at 12% +/- 2%, indicating that expression of the amphiphysin2 construct did not affect NTR1 internalization (Figure 27).

Fluo-TF was also able to internalize in the presence of GFP-A-SH3 (Figure 22B and 22B') and there was no sigificant difference between the percentage of total GFP-A-SH3 cells co-labeled with fluo-NT (58.6 +/- 3.3%) and the percentage of total GFP positive cells co-labeled with fluo-TF (57.4 +/- 3.3%), as depicted in Figure 28.

3.6.2 Effect of overexpressing the GFP-tagged SH3 domain of amphiphysin on NTR1 internalization in HEK 293 cells

The distribution of GFP-A-SH3 labeling was also homogeneous throughout dually transfected HEK 293 cells (Figure 22C). In cells expressing GFP-A-SH3, fluo-NT labeling was able to withstand treatment with acid wash (Figure 22C'), and therefore, was entirely intracellular. In HEK 293 cells, as per Figure 29, the percentages of GFP-A-SH3 cells co-labeled with fluo-NT in the TOTAL and ACID WASH conditions were identical, at 41% +/- 5% and 41 +/- 7%, respectively, indicating that the presence of the amphiphysin2 construct had no effect on NTR1 internalization.

Fluo-TF was also able to accumulate in an intracellular region in the presence of GFP-A-SH3 (data not shown), and the difference between the percentage of GFP-A-SH3 labeled cells that were positive for fluo-TF staining (69 +/- 6%) and the percentage of HEK 293 cells transfected with GFP alone that co-labeled fluo-TF (80 +/- 2%) was not statistically significant, as illustrated in Figure 30.

A summary of experimental results is presented in Table 3 and in cartoon form in Figures 31 and 32.

Figure 14 – Internalization of fluo-NT and fluo-TF in COS-7 and HEK 293 cells. (A) COS-7 cells transfected with NTR1 and incubated with 20 nM fluo-NT for 45 minutes at 37°C display a concentrated fluo-NT accumulation in a region of the cell that is close to the nucleus. (B) Subsequent to hypertonic acid wash, it becomes obvious that fluo-NT is gathered inside the cell. (C) Untransfected COS-7 cells incubated with 25 ug/ml of fluo-TF for 20 minutes at 37°C accumulate fluo-TF near the center of the cell. (D) HEK 293 cells transfected with NTR1 and incubated with 20 nM fluo-NT for 45 minutes at 37°C exhibit a different pattern of labeling compared to COS-7 cells in that staining is more diffuse throughout the cytoplasm of the cell. (E) Following hypertonic acid wash, HEK 293 cells do not exhibit the distinct, concentrated fluo-NT accumulation as seen in COS-7 cells (Figures 14A and 14B); although clearly intracellular, labeling forms multiple fluorescent "hot spots" dispersed throughout the cell. (F) Untransfected HEK 293 cells incubated with fluo-TF at 37°C for 20 minutes accumulate the ligand in a discrete spot next to the nucleus about midway from the plasma membrane to the nucleus.

Figure 14

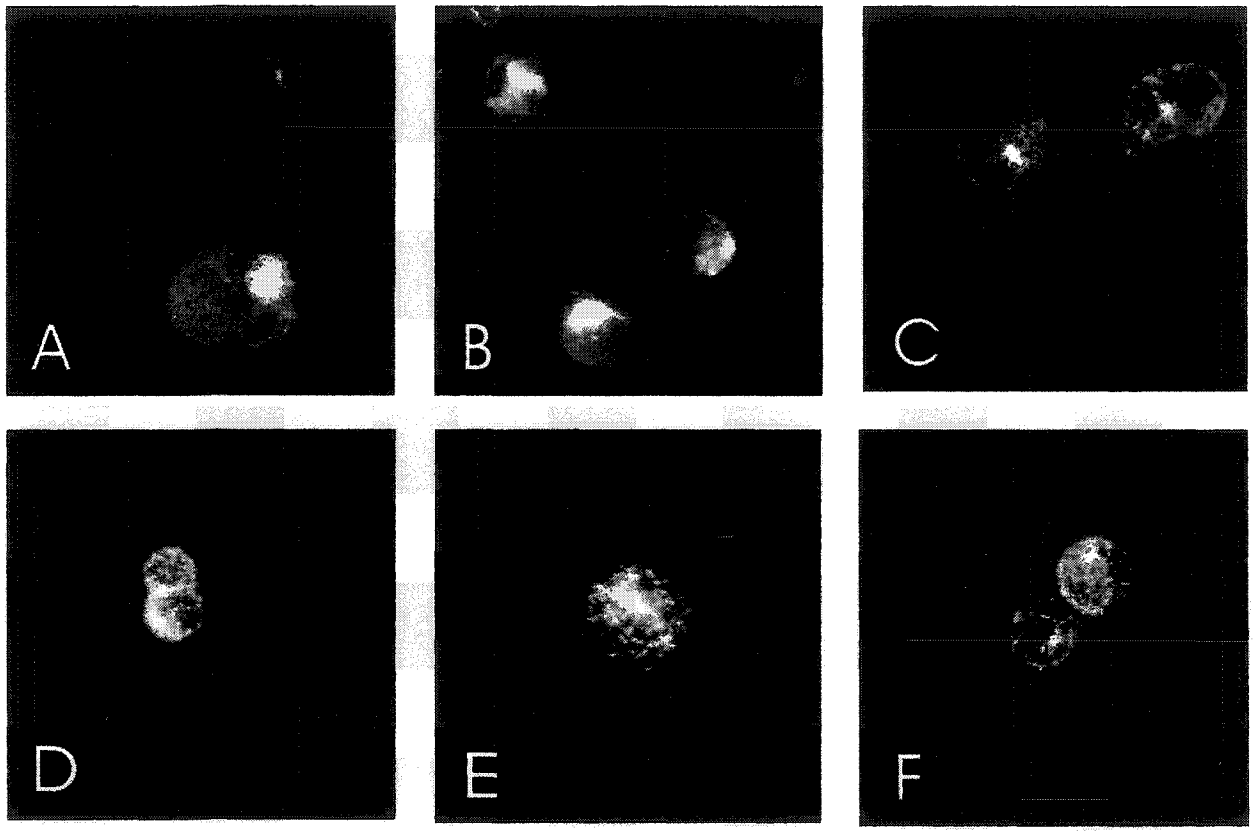


Figure 15 - Effect of mutant beta-arrestin (V53D) on fluo-NT internalization in COS-7 and HEK 293 cells. (A) COS-7 cells co-transfected with NTR1 and a mutant beta-arrestin (V53D) incubated with 20 nM fluo-NT for 45 minutes at 37°C accumulate a bright fluo-NT labeling on the cell surface. (B) Following hypertonic acid wash, surface-bound fluo-NT labelling is completely lost. (C) HEK 293 cells co-transfected with NTR1 and beta-arrestin (V53D) incubated with 20 nM fluo-NT for 45 minutes at 37°C accumulate surface-bound fluo-NT labeling which is not resistant to hypertonic acid wash and is completely washed away as in (D). Note the brighter labeling of fluo-NT binding in COS-7 cells co-transfected with V53D compared to that in HEK 293 cells (Figures 15A and 15C).

Figure 15

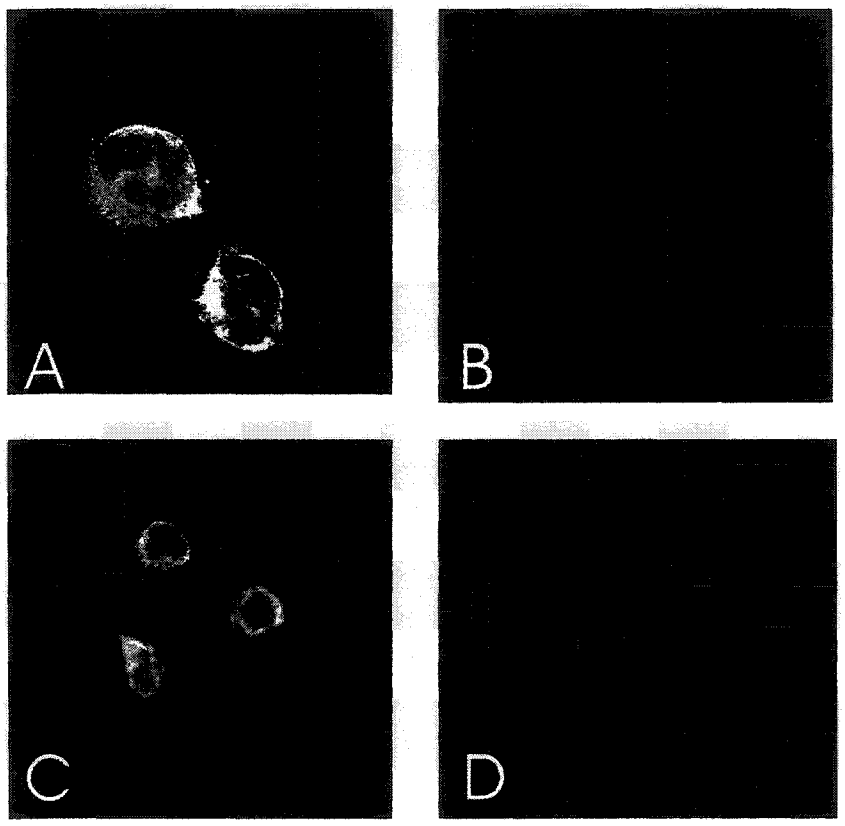


Figure 16 –Effect of mutant dynamin (K44A) on fluo-NT and fluo-TF internalization in COS-7 and HEK 293 cells. (A) COS-7 cells co-transfected with NTR1 and a mutant dynamin (K44A) incubated with 20 nM fluo-NT for 45 minutes at 37°C accumulate a bright surface labeling. (B) Subsequent to hypertonic acid wash, fluo-NT labeling is washed out. (C) COS-7 cells singly transfected with dynamin K44A and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C gather ligand at the periphery of the cells. (D) HEK 293 cells co-transfected with NTR1 and a mutant dynamin (K44A) incubated with 20 nM fluo-NT for 45 minutes at 37°C accumulate bright peripheral labeling that encircles the cell. (E) Subjection to hypertonic acid strips away the surface-bound labeling. (F) HEK 293 cells transfected with dynamin (K44A) incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C do not internalize ligand and accumulate it extracellularly.

Figure 16

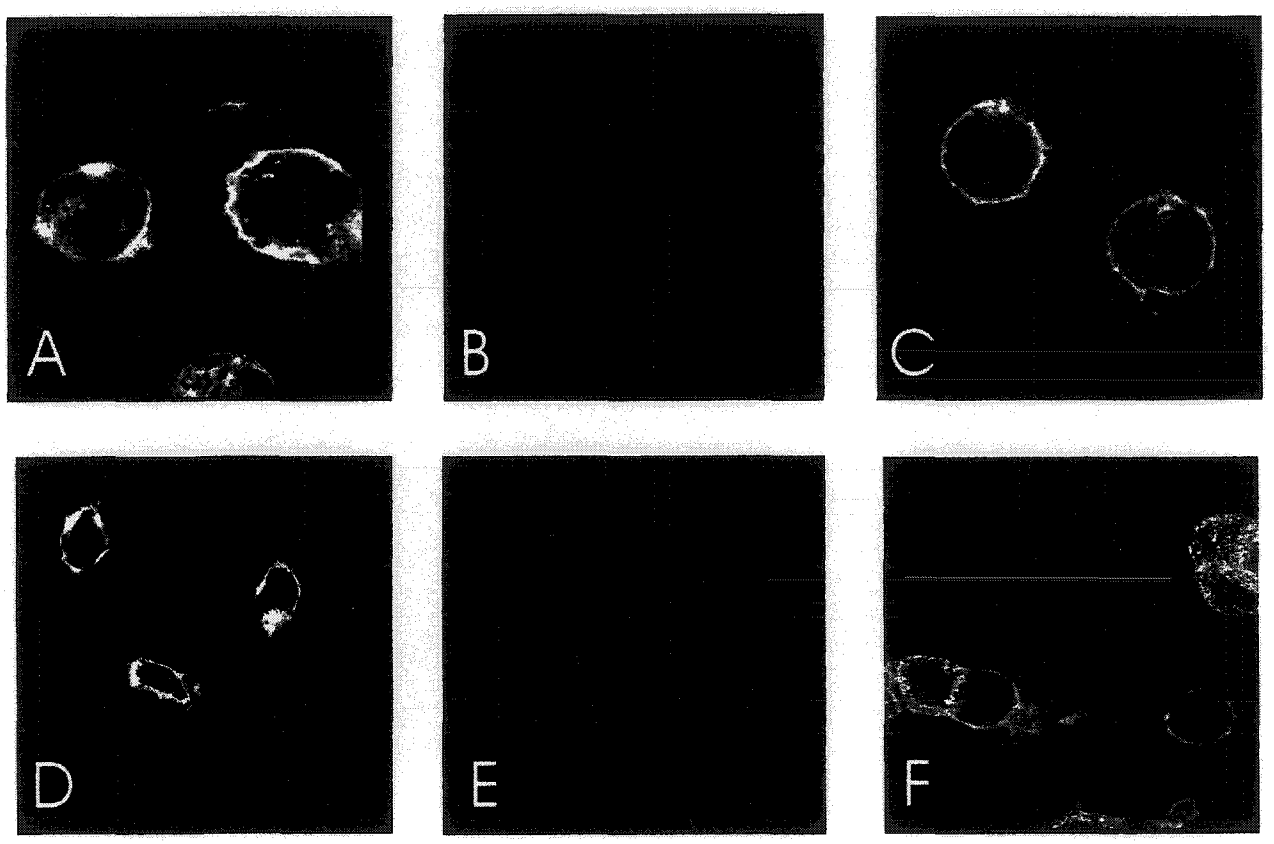


Figure 17 - Effect of GFP on fluo-NT internalization in COS-7 and HEK 293 cells and fluo-TF internalization in HEK 293 cells. (A) COS-7 cell co-transfected with NTR1 and GFP, incubated with 20 nM fluo-NT for 45 minutes at 37°C, and subjected to hypertonic acid wash. GFP staining of COS-7 cell is found homogeneously throughout the cell. (A') Same cell stained for fluo-NT accumulates ligand in a discrete spot close to the plasma membrane. (B) HEK 293 cells co-transfected with NTR1 and GFP, incubated with 20 nM fluo-NT for 45 minutes at 37°C and subjected to hypertonic acid wash. GFP staining of HEK 293 cell. (B') Same cell stained for fluo-NT exhibits a distinct spot of fluo-NT near the middle of the cell as well as bright staining adjacent to the plasma membrane. (C) HEK 293 cells singly transfected with GFP and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C. GFP staining of HEK 293 cells. (C') Fluo-TF staining of HEK 293 cells. Note the intracellular accumulation of fluo-TF in the cell co-labeled with GFP (arrow).

Figure 17

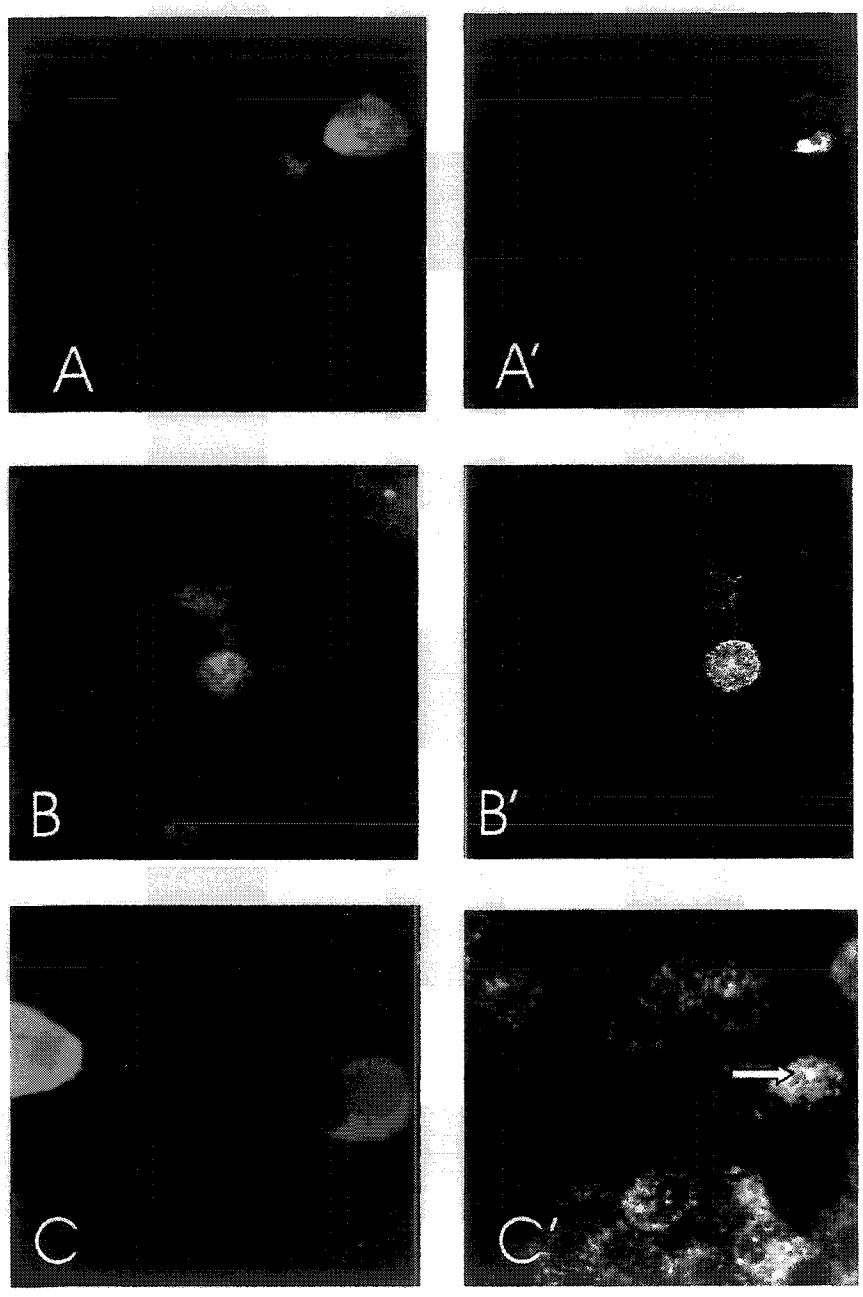


Figure 18 - Effect of full length intersectin on fluo-NT internalization in COS-7 and HEK 293 cells and fluo-TF internalization in HEK 293 cells. (A) COS-7 cells co-transfected with NTR1 and GFP - INT and incubated with 20 nM fluo-NT for 45 minutes at 37°C. GFP - INT staining of COS-7 cell accumulate in puncta throughout the cell. (A') Same cell stained for fluo-NT accumulates plasma-membrane bound ligand. (B) COS-7 cells subjected to hypertonic acid wash; staining for GFP - INT. (B') Same cells stained for fluo-NT exhibit non-specific labeling. (C) COS-7 cell singly transfected with GFP – INT and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C. GFP - INT staining of COS-7 cell. (C') Same cell (arrow) stained for fluo-TF. Note the peripheral staining of fluo-TF in the cell co-expressing GFP – INT. (D) HEK 293 cells co-transfected with NTR1 and GFP - INT, incubated with 20 nM fluo-NT for 45 minutes at 37°C, and subjected to hypertonic acid wash. (D') Same cell (arrow) labeled with fluo-NT. Note the acid-resistant labeling of fluo-NT even in the presence of GFP – INT, and the similarity of fluo-NT staining between the cell co-labeled with GFP – INT and the adjacent cells that do not co-label with GFP – INT.

Figure 18

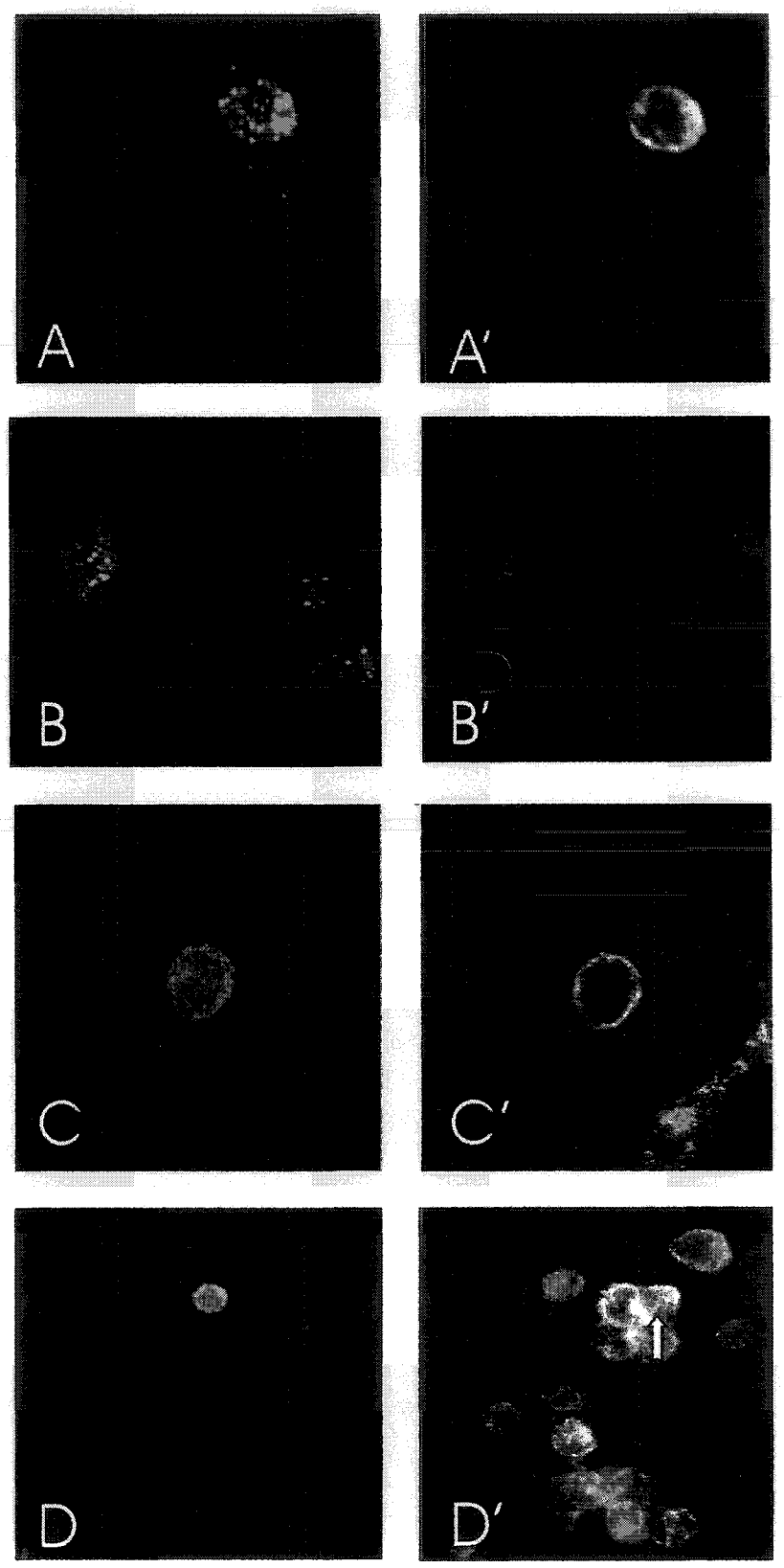


Figure 19 – Effect of the SH3 domains of intersectin on internalization of fluo-NT in COS-7 and HEK 293 cells. (A) COS-7 cells co-transfected with the SH3 domains of intersectin tagged to GFP (GFP-I-SH3) and NTR1 and incubated with 20 nM fluo-NT for 45 minutes at 37°C. GFP-I-SH3 staining of COS-7 cells is found homogeneously throughout the cell. (A') Same cell stained for fluo-NT. Note the intense, bright peripheral labeling of fluo-NT in the presence of GFP-I-SH3. Following hypertonic acid wash, GFP-I-SH3 labeling remains distributed throughout the cell (B), but fluo-NT staining is completely washed away, as evidenced by the lack of specific labeling after acid wash (B'). (C) HEK 293 cells co-transfected with GFP-I-SH3 and NTR1 and incubated with 20 nM fluo-NT for 45 minutes at 37°C. HEK 293 cell staining for GFP-I-SH3. (C') Same cell stained for fluo-NT accumulates ligand in a distinct spot adjacent to the nucleus. Subsequent to hypertonic acid wash, it is clear that the cell co-labeled with GFP-I-SH3 (D) is able to effectively internalize fluo-NT (D'), unlike its COS-7 cell counterpart.

Figure 19

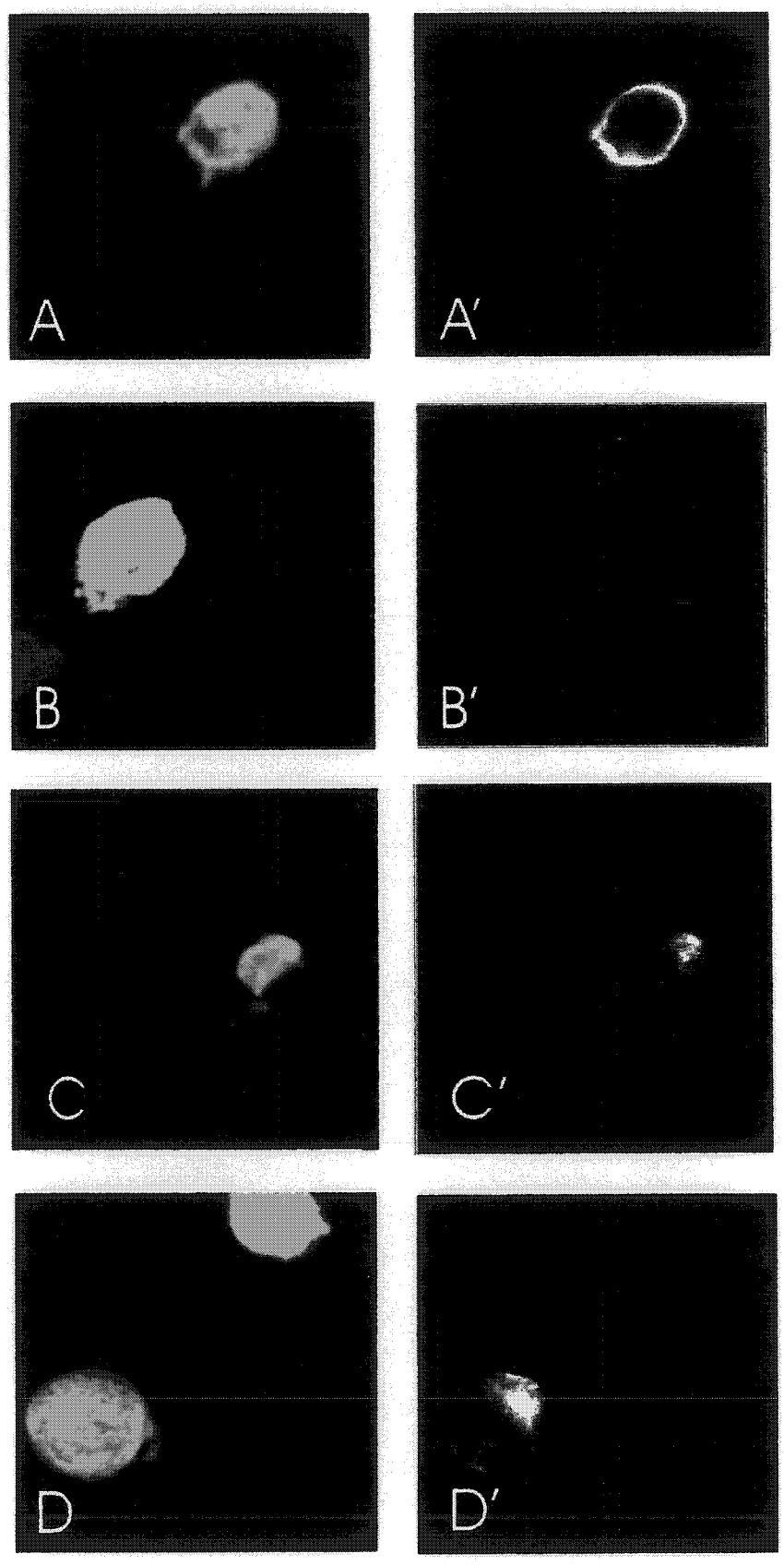


Figure 20 – Effect of the SH3 domains of intersectin on internalization of fluo-TF in HEK 293 cells. (A) HEK 293 cells transfected with GFP-I-SH3 and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C. GFP-I-SH3 accumulates throughout the cell. (A') Same cell labeled for fluo-TF illustrates the ligand's inability to enter the cell in the presence of GFP-I-SH3. Note that expression of GFP-I-SH3 in the same cell line did not hinder the internalization of fluo-NT (Figure 20D and D'), indicating that fluo-NT and fluo-TF internalize through distinct mechanisms with regards to the role of the SH3 domain of intersectin.

Figure 20

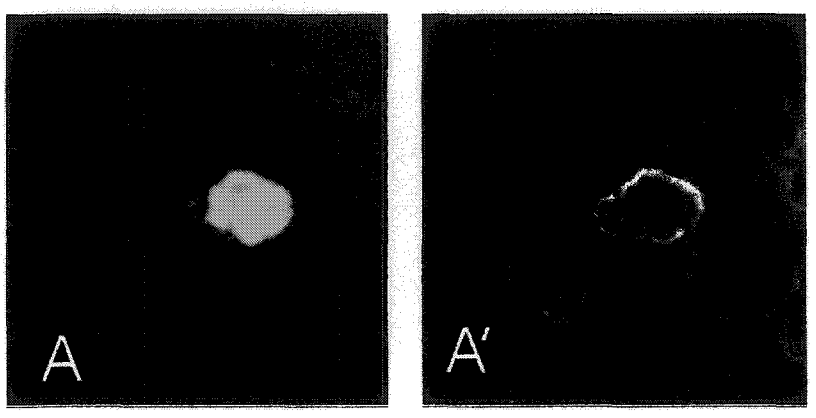


Figure 21 - Effect of the EH domains of intersectin on internalization of fluo-NT in COS-7 and HEK 293 cells and fluo-TF in HEK 293 cells. (A) COS-7 cells co-transfected with the EH domains of intersectin tagged to GFP (GFP-I-EH) and NTR1, incubated with 20 nM fluo-NT for 45 minutes at 37°C, and subjected to hypertonic acid wash. GFP-I-EH staining of COS-7 cell is found throughout the cell. (A') Same cell stained for fluo-NT depicts the accumulation of ligand in a distinct intracellular location resistant to acid wash. (B) HEK 293 cells co-transfected with GFP-I-EH and NTR1, incubated with 20 nM fluo-NT for 45 minutes at 37°C, and subjected to hypertonic acid wash. GFP-I-EH staining of COS-7 cell. (B') Same cell (arrow) stained for fluo-NT exhibits an intracellular labeling of ligand that is resistant to acid wash. (C) HEK 293 cells singly transfected with GFP-I-EH and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C. GFP-I-EH staining of HEK 293 cell. (C') Same cell labelled with fluo-TF displays the ligand's inability to enter the cell in the presence of GFP-I-EH.

Figure 21

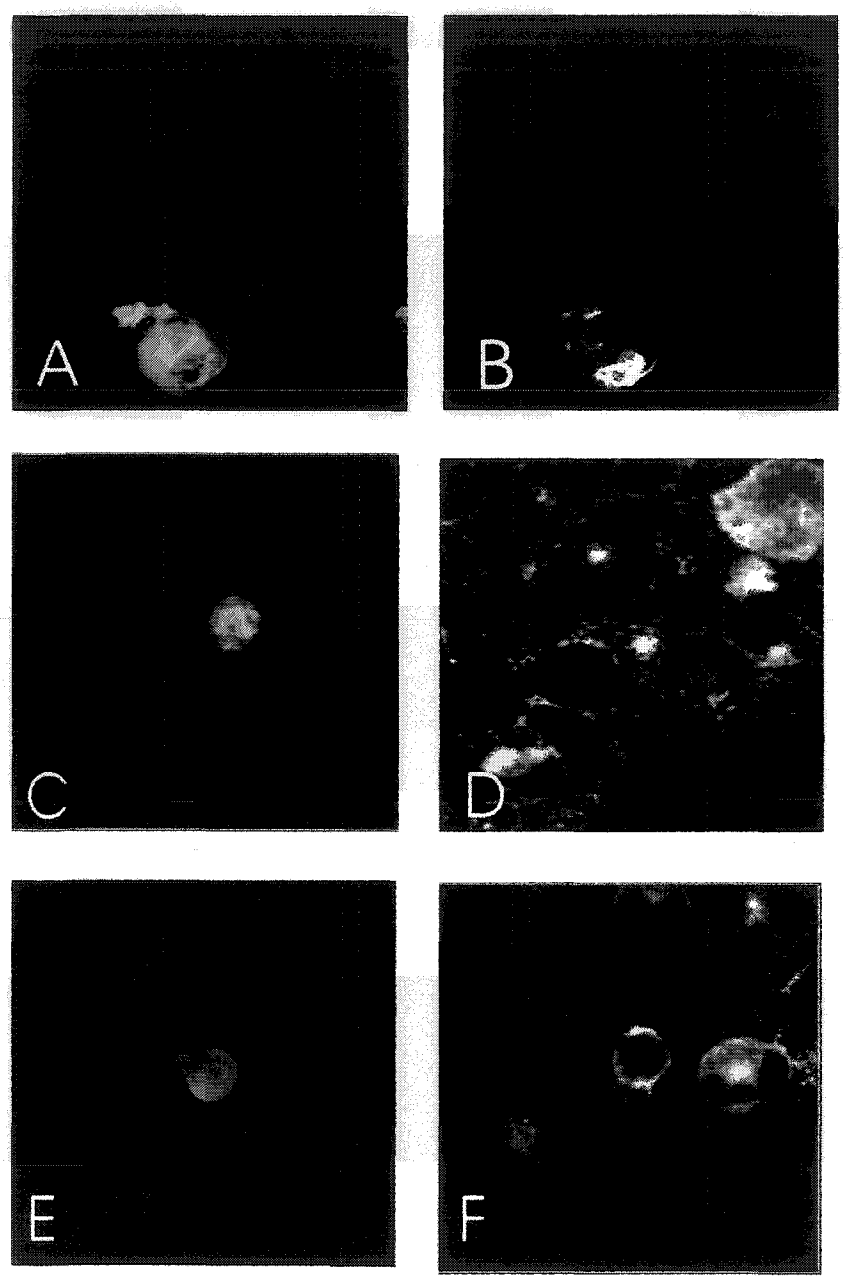


Figure 22 – Effect of the SH3 domain of amphiphysin2 on fluo-NT internalization in COS-7 and HEK 293 cells and fluo-TF in COS-7 cells. (A) COS-7 cells co-transfected with the SH3 domain of amphiphysin 2 tagged to GFP (GFP-A-SH3) and NTR1, incubated with 20 nM fluo-NT for 45 minutes at 37°C and subjected to hypertonic acid wash. GFP-A-SH3 staining of COS-7 cell distributes throughout the entire cell. (A') Same cell stained for fluo-NT depicts the intracellular localization of ligand despite the presence of GFP-A-SH3. (B) COS-7 cells singly transfected with GFP-A-SH3 and incubated with 25 ug/ml fluo-TF for 20 minutes at 37°C. GFP-A-SH3 staining of HEK 293 cell. (B') Same cell (arrow) labeled with fluo-TF, which accumulates intracellularly. (C) HEK 293 cells incubated with 20 nM fluo-NT for 45 minutes at 37°C and subjected to hypertonic acid wash. Staining for GFP-A-SH3. (C') Same cell stained for fluo-NT gathers ligand in a distinct, intracellular spot.

Figure 22

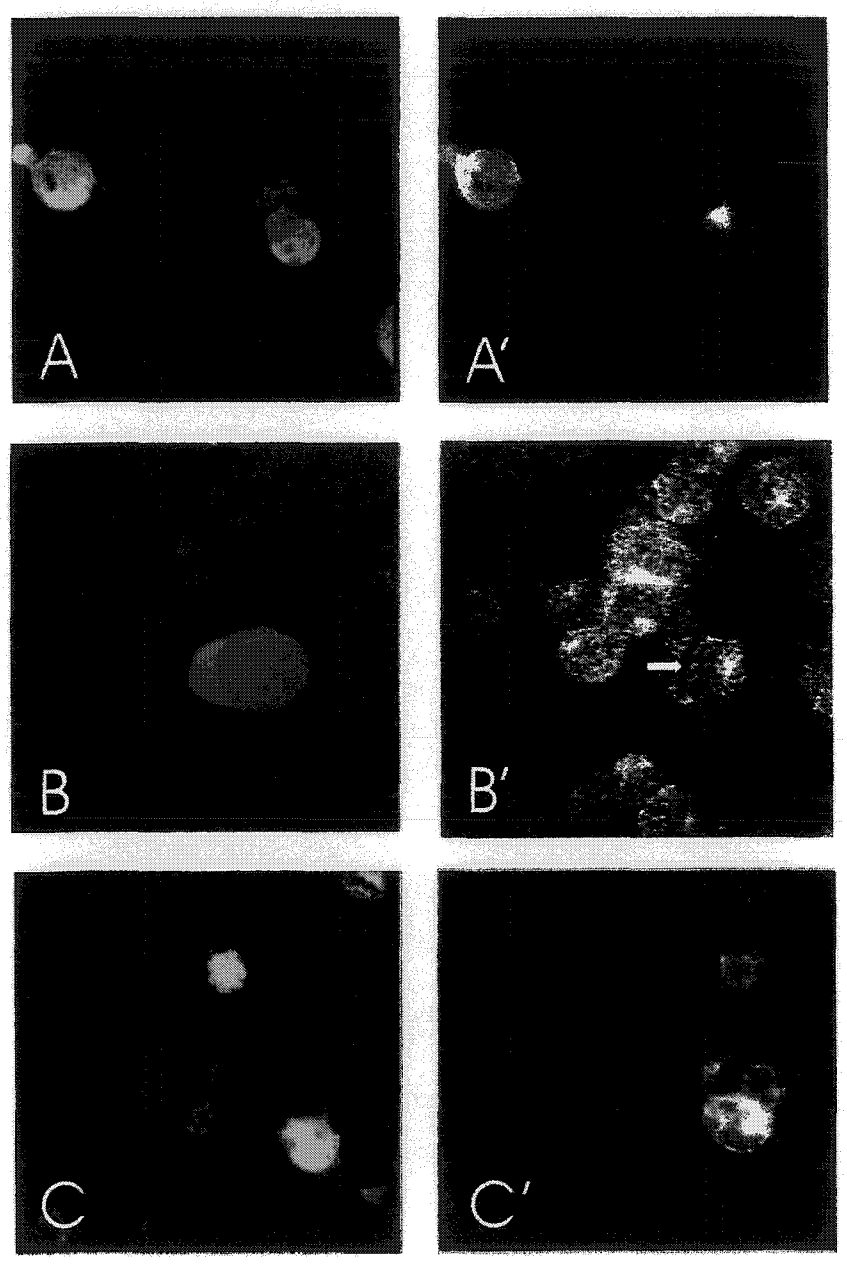
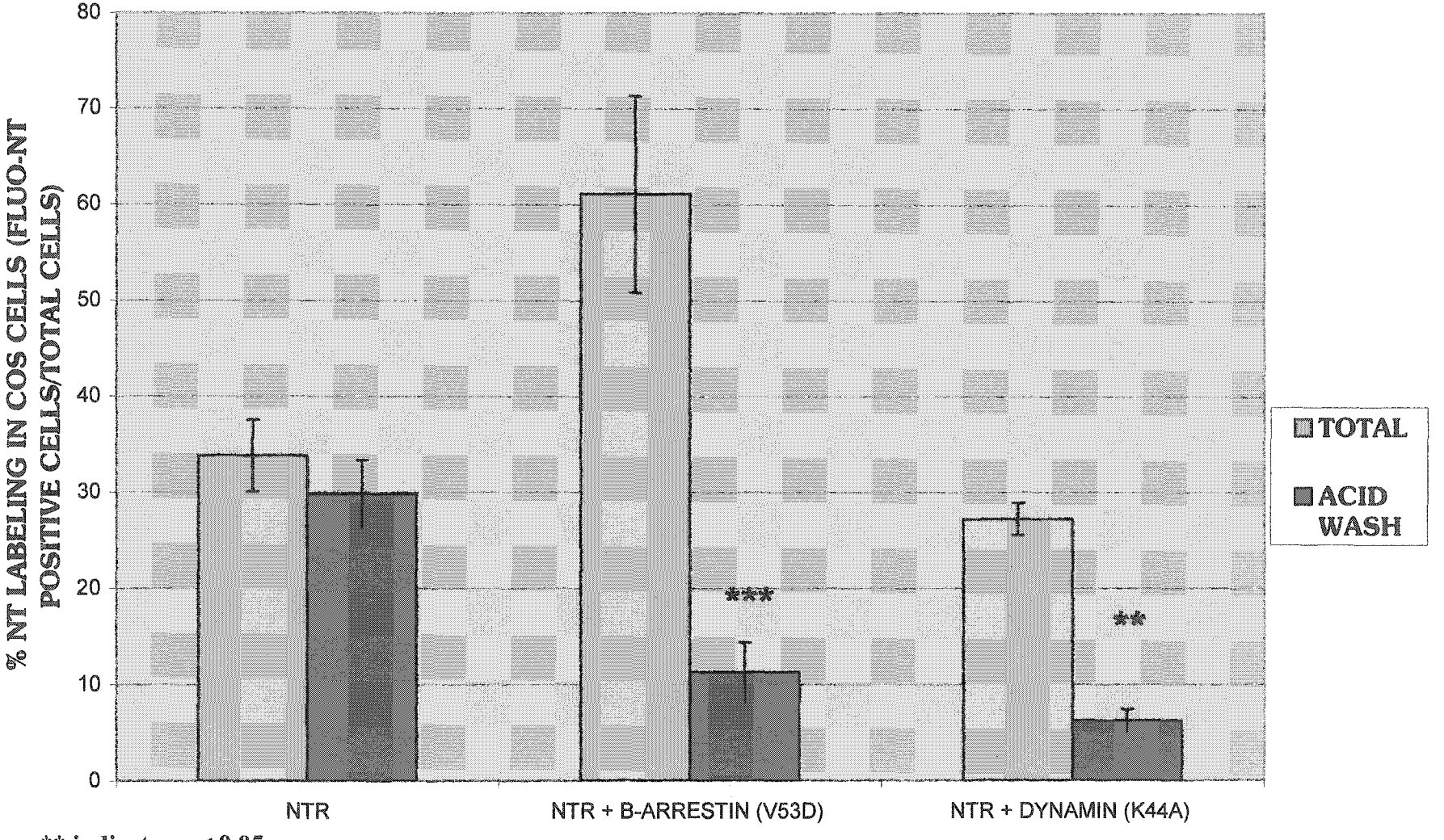


Figure 23 - Internalization of fluo-NT in COS-7 cells transfected or not with mutant beta-arrestin V53D or mutant dynamin K44A. When NTR1 was transfected into COS-7 cells, 34 % of the total amount of cells were labeled with fluo-NT in the TOTAL condition. As this value was not significantly different from the ACID WASH condition, fluo-NT that was observed was internalized ligand. COS-7 cells co-transfected with NTR1 and mutant dynamin K44A exhibited 27% fluo-NT binding in the TOTAL condition, a value that decreased significantly in the ACID WASH condition, where the percentage of fluo-NT labeled cells was only 6% ($p < 0.05$; n=6). Similarly, when COS-7 cells were co-transfected with NTR1 and the mutant beta-arrestin V53D, the percentage of fluo-NT cells was 61% in the TOTAL condition, while in the ACID WASH condition, only 11% of COS-7 cells were fluo-NT positive ($p < 0.01$; n=5). These results propose that beta-arrestin and dynamin are involved in the internalization of fluo-NT in COS-7 cells.

FIGURE 23 % FLUO-NT LABELING IN COS CELLS

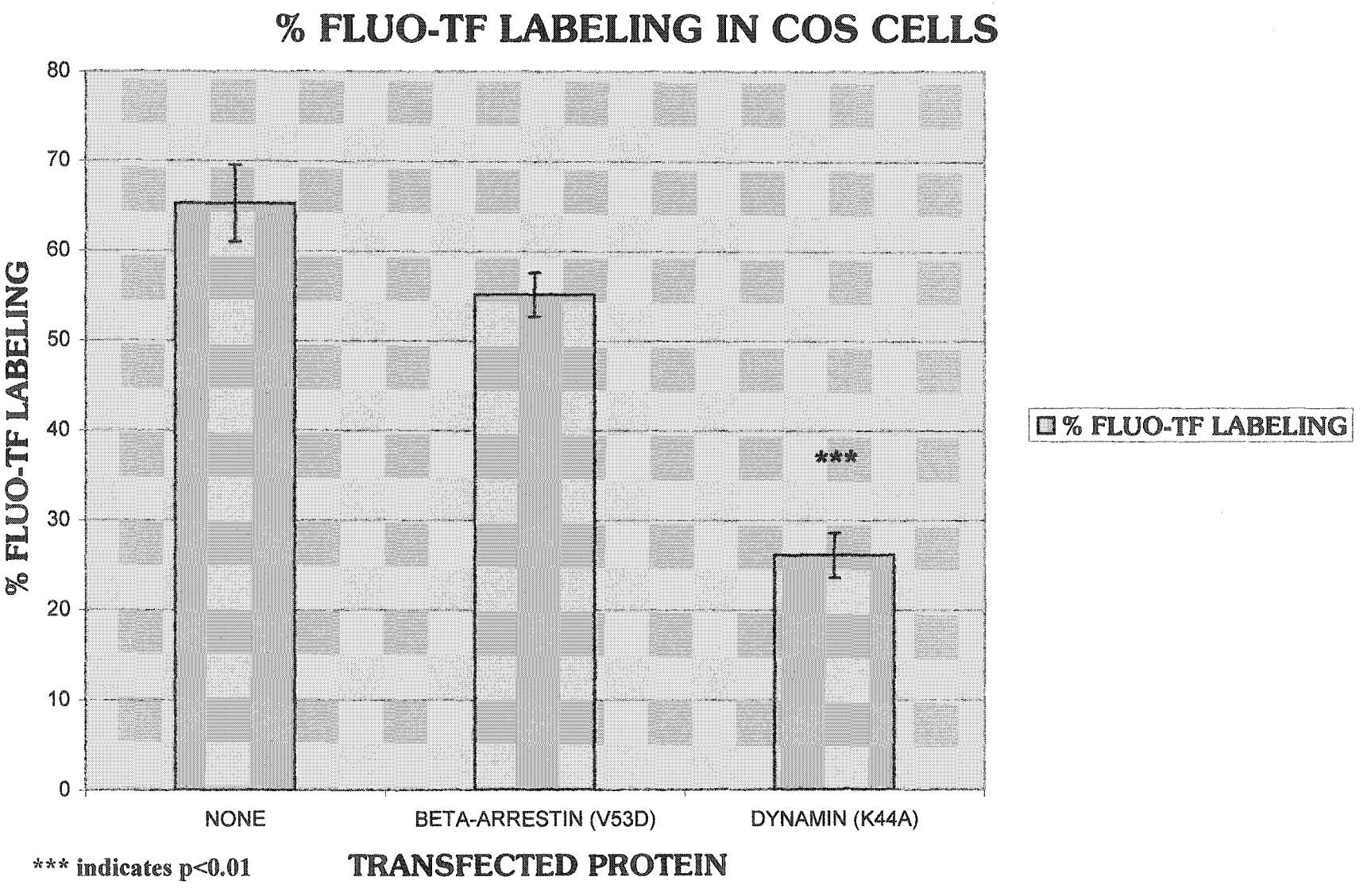


** indicates $p < 0.05$

*** indicates $p < 0.01$

Figure 24 - Fluo-TF internalization in COS-7 cells transfected or not with mutant beta-arrestin V53D or mutant dynamin K44A. In untransfected HEK 293 cells, 64% of total cells internalize fluo-TF after 20 minutes of incubation at 37°C. In COS-7 cells transfected with the mutant beta-arrestin V53D, 55% of COS-7 cells internalize fluo-TF, a value that is not significantly different from the percentage of cells that internalize fluo-TF in nontransfected COS-7 cells. In COS-7 cells transfected with mutant dynamin K44A, only 25% of total COS-7 cells were able to internalize fluo-TF, a difference that is statistically significant when compared to the value in untransfected cells ($p < 0.01$; $n = 10$). These results indicate that dynamin, but not beta-arrestin, is involved in the internalization of fluo-TF in COS-7 cells.

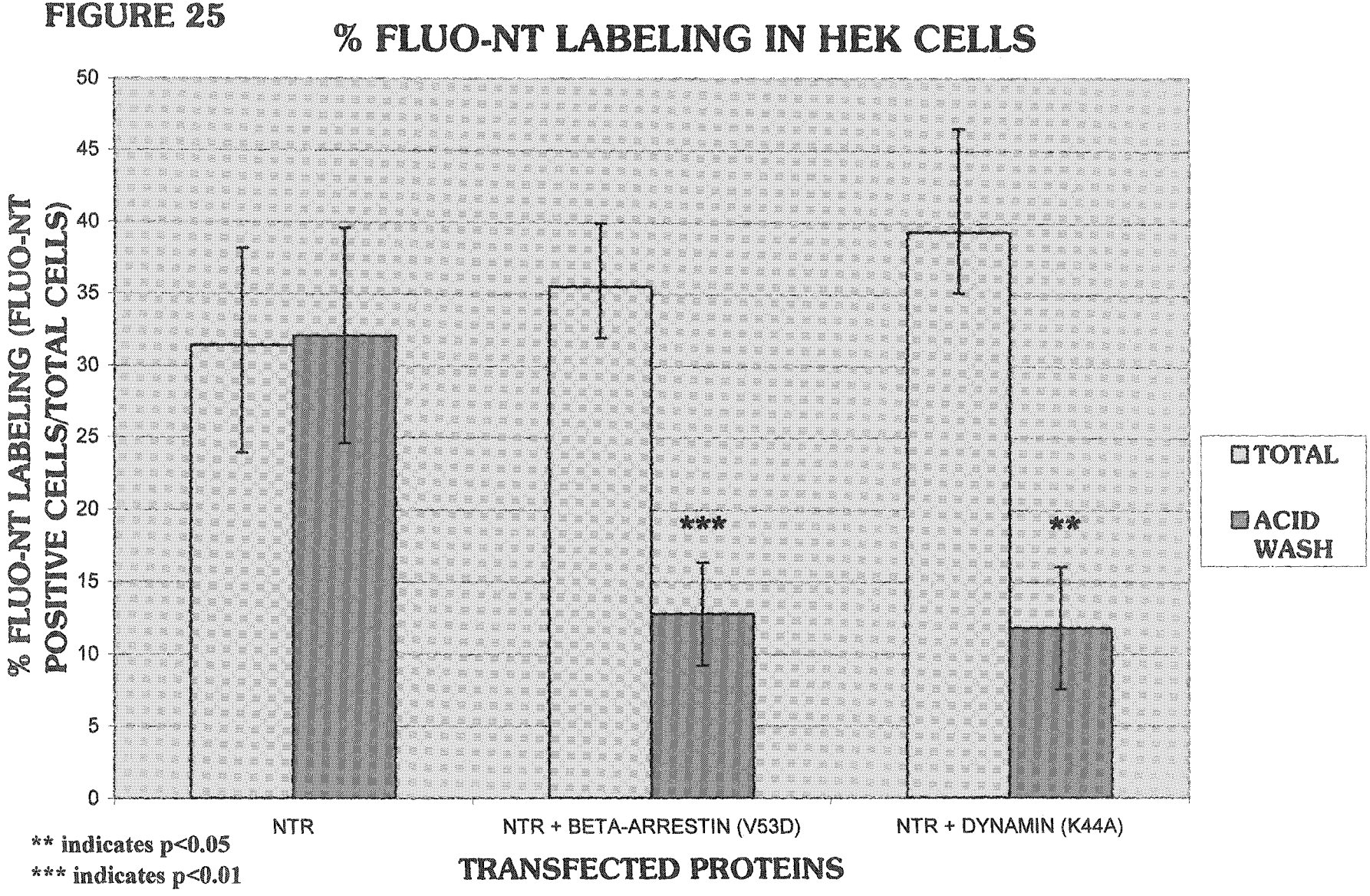
FIGURE 24



*** indicates p<0.01

Figure 25 - Internalization of fluo-NT in HEK 293 cells transfected or not with mutant beta-arrestin V53D or mutant dynamin K44A. When NTR1 is transfected into HEK 293 cells, 34% of the total amount of cells are labeled with fluo-NT in the TOTAL condition. As this value was not significantly different in the ACID WASH condition, fluo-NT that is observed is internalized ligand. HEK 293 cells co-transfected with NTR1 and mutant dynamin K44A exhibited 39% of fluo-NT binding in the TOTAL condition, a value that decreased significantly in the ACID WASH condition, where the percentage of fluo-NT labeled cells was only 12% ($p<0.05$; $n = 4$). Similarly, when HEK 293 cells were co-transfected with NTR1 and the mutant beta-arrestin V53D, the percentage of fluo-NT cells was 35% in the TOTAL condition, while in the ACID WASH condition, only 13% of HEK 293 cells were fluo-NT positive ($p<0.01$, $n=3$). These results suggest that both beta-arrestin and dynamin play a role in the internalization of fluo-NT in HEK 293 cells.

FIGURE 25

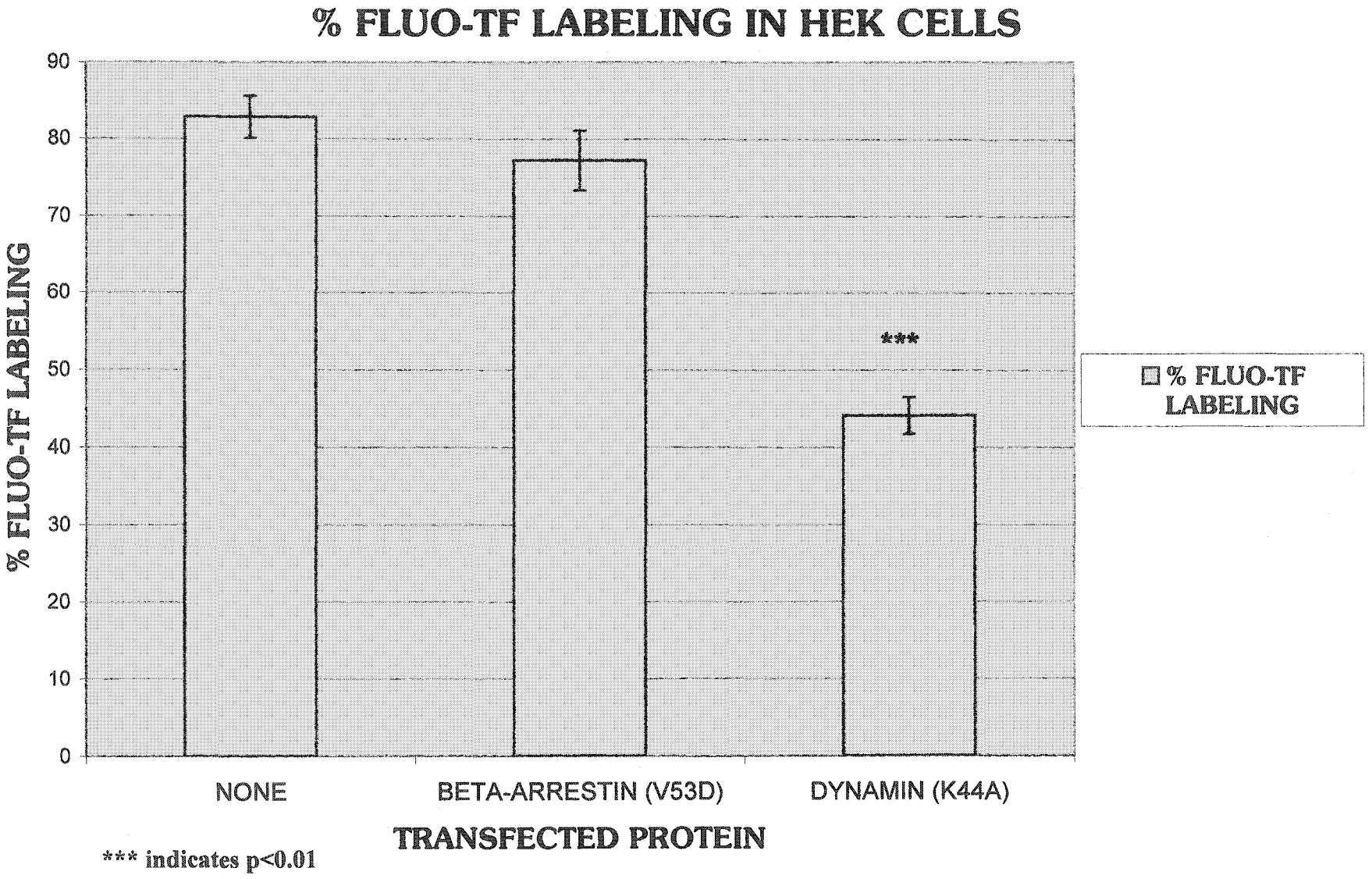


** indicates p<0.05

*** indicates p<0.01

Figure 26 - Fluo-TF internalization in HEK 293 cells transfected or not with mutant beta-arrestin V53D or mutant dynamin K44A. In untransfected HEK 293 cells, 80% of total cells internalize fluo-TF after 20 minutes of incubation at 37°C. In HEK 293 cells transfected with the mutant beta-arrestin V53D, 77% of HEK 293 cells internalize fluo-TF, a value that is not significantly different from the percentage of cells that internalize fluo-TF in nontransfected HEK 293 cells. In HEK 293 cells transfected with mutant dynamin K44A, only 42% of total HEK 293 cells were able to internalize fluo-TF, a difference that is statistically significant when compared to the value in untransfected cells ($p<0.01$; $n= 10$). These results indicate that dynamin, but not beta-arrestin, is involved in the internalization of fluo-NT in HEK 293 cells.

FIGURE 26



*** indicates p<0.01

Figure 27 - Internalization of fluo-NT in the presence of GFP or GFP-tagged constructs in COS-7 cells. In COS-7 cells co-transfected with NTR1 and GFP, the percentage of total GFP positive cells that co-labeled with fluo-NT was 20% in the TOTAL condition, and 13% in the ACID WASH condition, the difference between the two conditions being not statistically significant. In COS-7 cells co-transfected with NTR1 and GFP – INT, the percentage of GFP – INT positive cells that co-labeled with fluo-NT was 28%, a value that decreased significantly under ACID WASH conditions to 11% ($p<0.01$, $n=12$). Similarly, in COS-7 cells co-transfected with NTR1 and GFP-I-SH3, 31% of total GFP-I-SH3 cells co-labeled with fluo-NT in the TOTAL condition, but only 12% did under ACID WASH conditions, indicating that there was a statistically significant difference between TOTAL and ACID WASH conditions ($p<0.01$, $n=18$). The percentage of GFP-I-EH positive cells that co-label with fluo-NT in COS-7 cells co-transfected with NTR1 and GFP-I-EH in the TOTAL condition is 20%, while in the ACID WASH this value was 17%, a result that does not differ significantly from the TOTAL condition. In COS-7 cells co-transfected with NTR1 and GFP-A-SH3, 16% of total GFP-A-SH3 positive cells co-labeled with fluo-NT in the TOTAL condition, while 13% of GFP-A-SH3 positive cells co-labeled with fluo-NT in the ACID WASH condition, a difference that is not statistically significant. These results indicate that dynamin and beta arrestin, but not intersectin or amphiphysin2, are involved in fluo-NT internalization in HEK 293 cells.

FIGURE 27

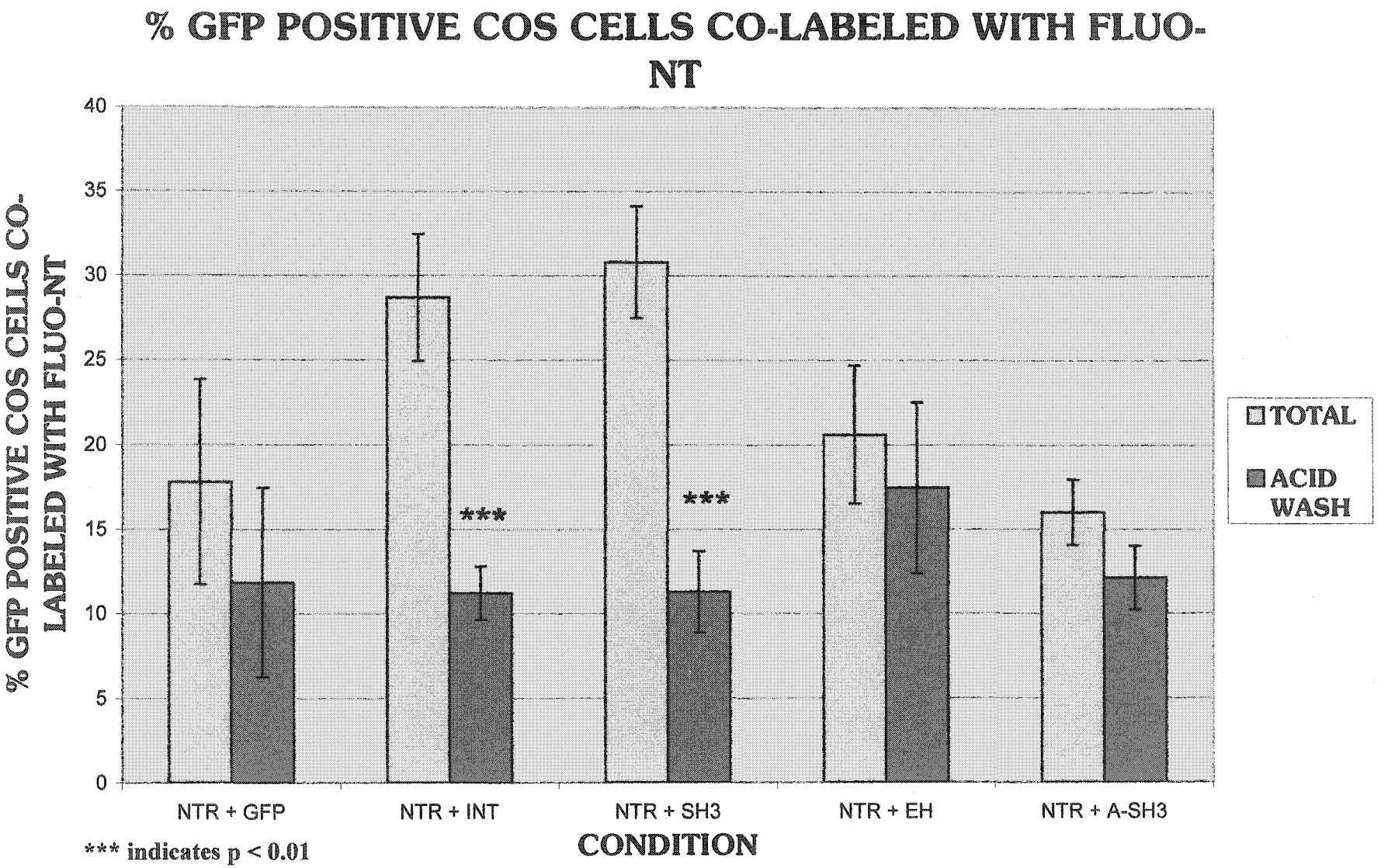

% GFP POSITIVE COS CELLS CO-LABELED WITH FLUO-NT
% GFP POSITIVE COS CELLS CO-LABELED WITH FLUO-NT
40
35
30
25
20
15
10
5
0
***
***
NTR + GFP
NTR + INT
NTR + SH3
NTR + EH
NTR + A-SH3
CONDITION
TOTAL
ACID WASH
*** indicates p < 0.01

Figure 28 - Internalization of fluo-TF in COS-7 cells in the presence of GFP or GFP-tagged constructs. The percentage of GFP positive cells that co-label with fluo-NT when COS-7 cells are transfected with GFP is 57%. In COS-7 cells transfected with GFP – INT, only 30% of GFP – INT positive cells co-labeled with fluo-TF, a value statistically significant from the value obtained when GFP alone is transfected into these cells ($p<0.01$; $n=19$). In COS-7 cells transfected with GFP-I-SH3, 42% of GFP-I-SH3 positive cells co-label with fluo-TF, a value that is again significantly different from that obtained in the control condition where GFP alone was transfected into COS-7 cells ($p<0.05$; $n=20$). COS-7 cells transfected with GFP-I-EH demonstrated that 67% of GFP-I-EH positive cells co-labeled with fluo-TF, a value that was not statistically different from the value in the GFP condition. In cells transfected with GFP-A-SH3, the percentage of total GFP-A-SH3 positive cells co-labeled with fluo-TF was 59%, a value not significantly different from the percentage of GFP positive cells co-labeled with fluo-TF in the control condition. These results suggest that intersectin, and in particular its SH3 domains, but not amphiphysin2, is involved in fluo-TF internalization in COS-7 cells.

FIGURE 28

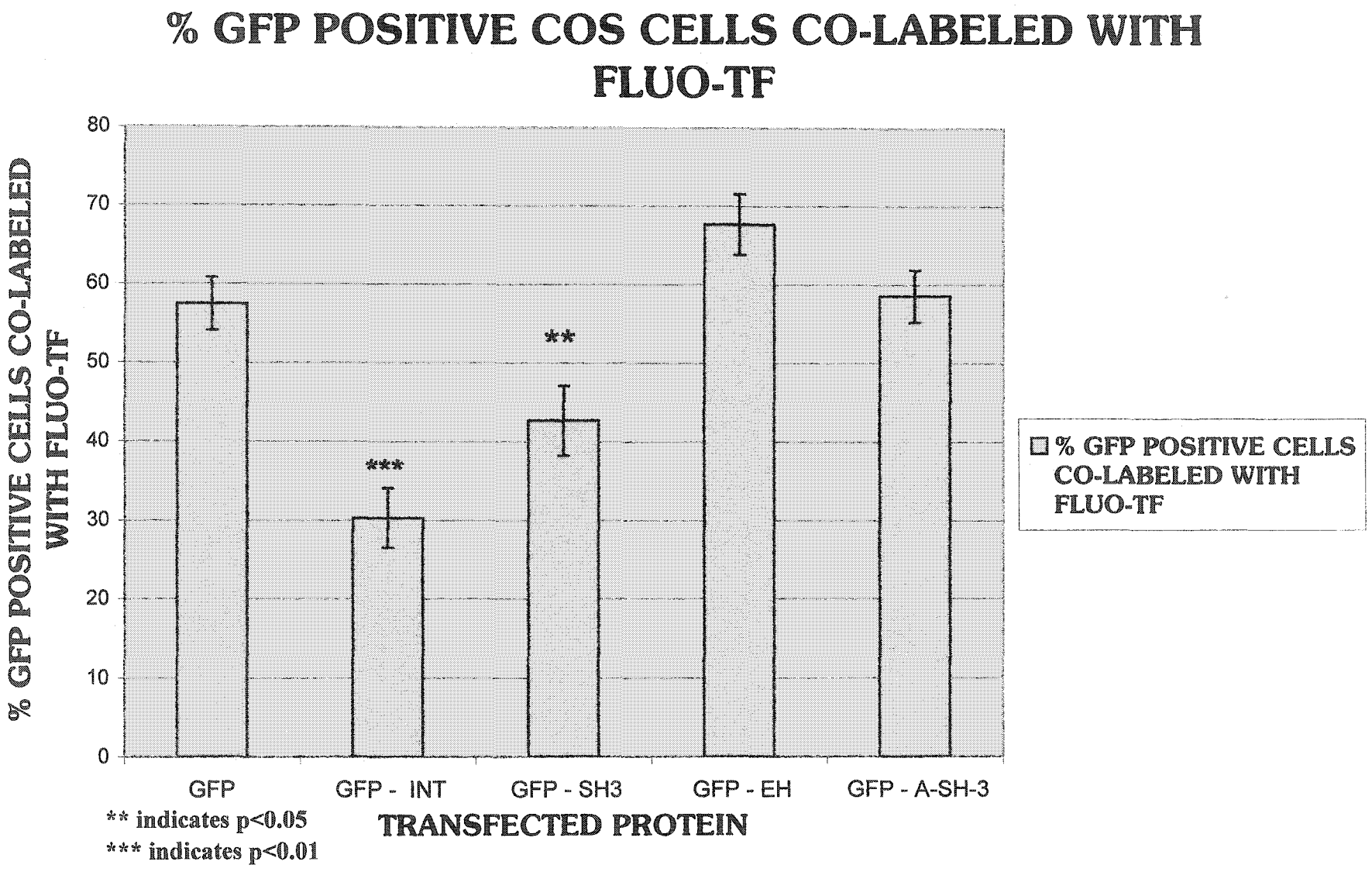

% GFP POSITIVE COS CELLS CO-LABELED WITH FLUO-TF
% GFP POSITIVE CELLS CO-LABELED WITH FLUO-TF
80
70
60
50
40
30
20
10
0
***
**
GFP
GFP - INT
GFP - SH3
GFP - EH
GFP - A-SH-3
TRANSFECTED PROTEIN
% GFP POSITIVE CELLS CO-LABELED WITH FLUO-TF
** indicates p<0.05
*** indicates p<0.01

Figure 29 - Internalization of fluo-NT in the presence of GFP or GFP-tagged constructs in HEK 293 cells. In HEK 293 cells co-transfected with NTR1 and GFP, the percentage of total GFP positive cells that co-labeled with fluo-NT was 36% in the TOTAL condition, and 37% in the ACID WASH condition, the difference between the two conditions not being statistically significant. In HEK 293 cells co-transfected with NTR1 and GFP – INT, the percentage of GFP – INT positive cells that co-labeled with fluo-NT was 57%, a value that decreased non-significantly under ACID WASH conditions to 51%. Similarly, in HEK 293 cells co-transfected with NTR1 and GFP-I-SH3, 42% of total GFP-I-SH3 cells co-labeled with fluo-NT in the TOTAL condition, while this value was 39% under ACID WASH conditions, indicating that there no statistically significant difference between TOTAL and ACID WASH conditions. The percentage of GFP-I-EH positive cells that co-labeled with fluo-NT in HEK 293 cells co-transfected with NTR1 and GFP-I-EH in the TOTAL condition was 47%, while in the ACID WASH condition this value was 41%, values that do not differ significantly from each other. In HEK 293 cells co-transfected with NTR1 and GFP-A-SH3, 41% of total GFP-A-SH3 positive cells co-labeled with fluo-NT in the TOTAL condition, while 41% of GFP-A-SH3 positive cells co-labeled with fluo-NT in the ACID WASH condition, a difference that was obviously not statistically significant. These results suggest that neither intersectin nor amphiphysin2 are involved in fluo-NT internalization in HEK 293 cells.

FIGURE 29

% GFP POSITIVE HEK CELLS CO-LABELED WITH FLUO-NT

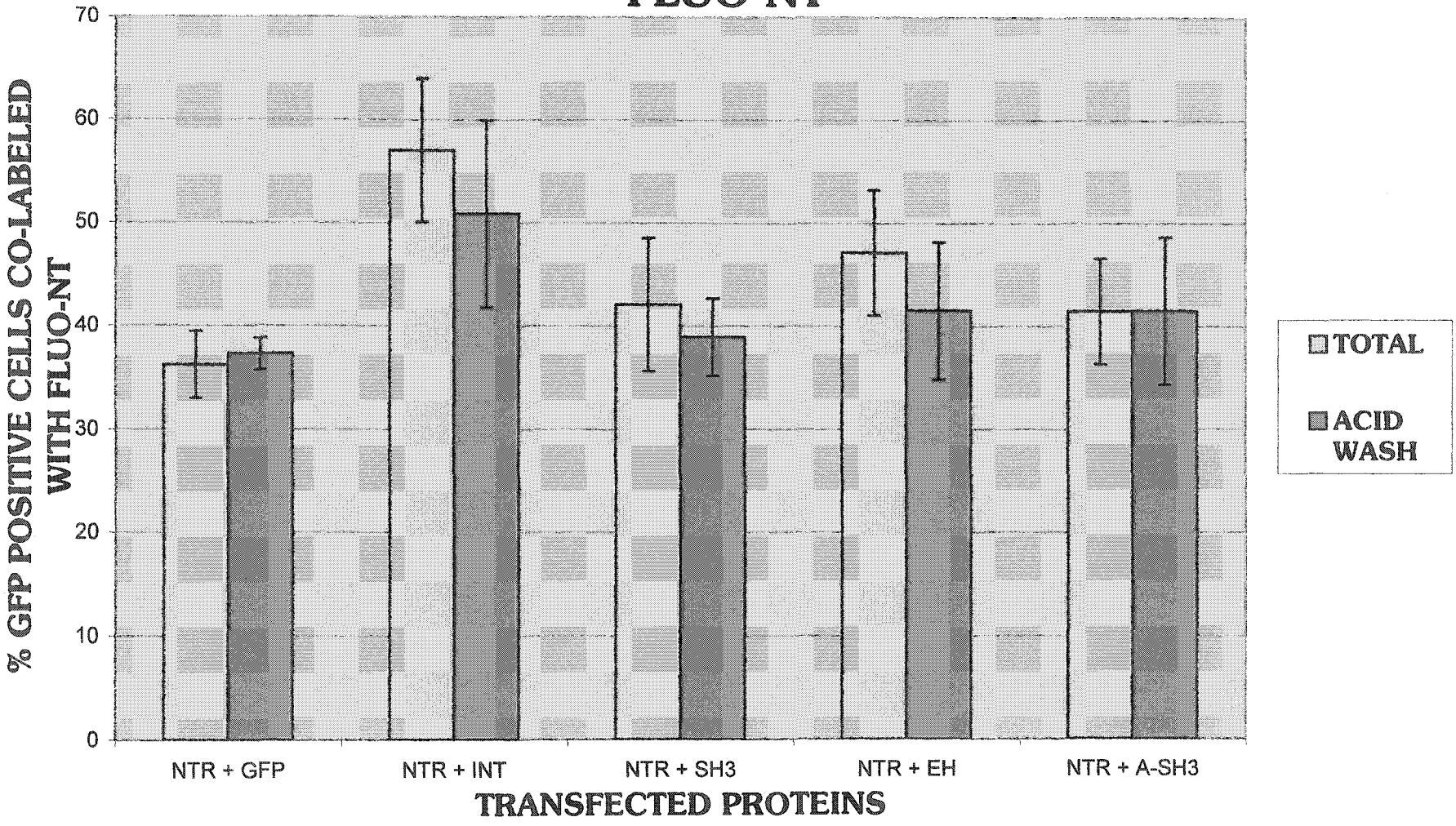
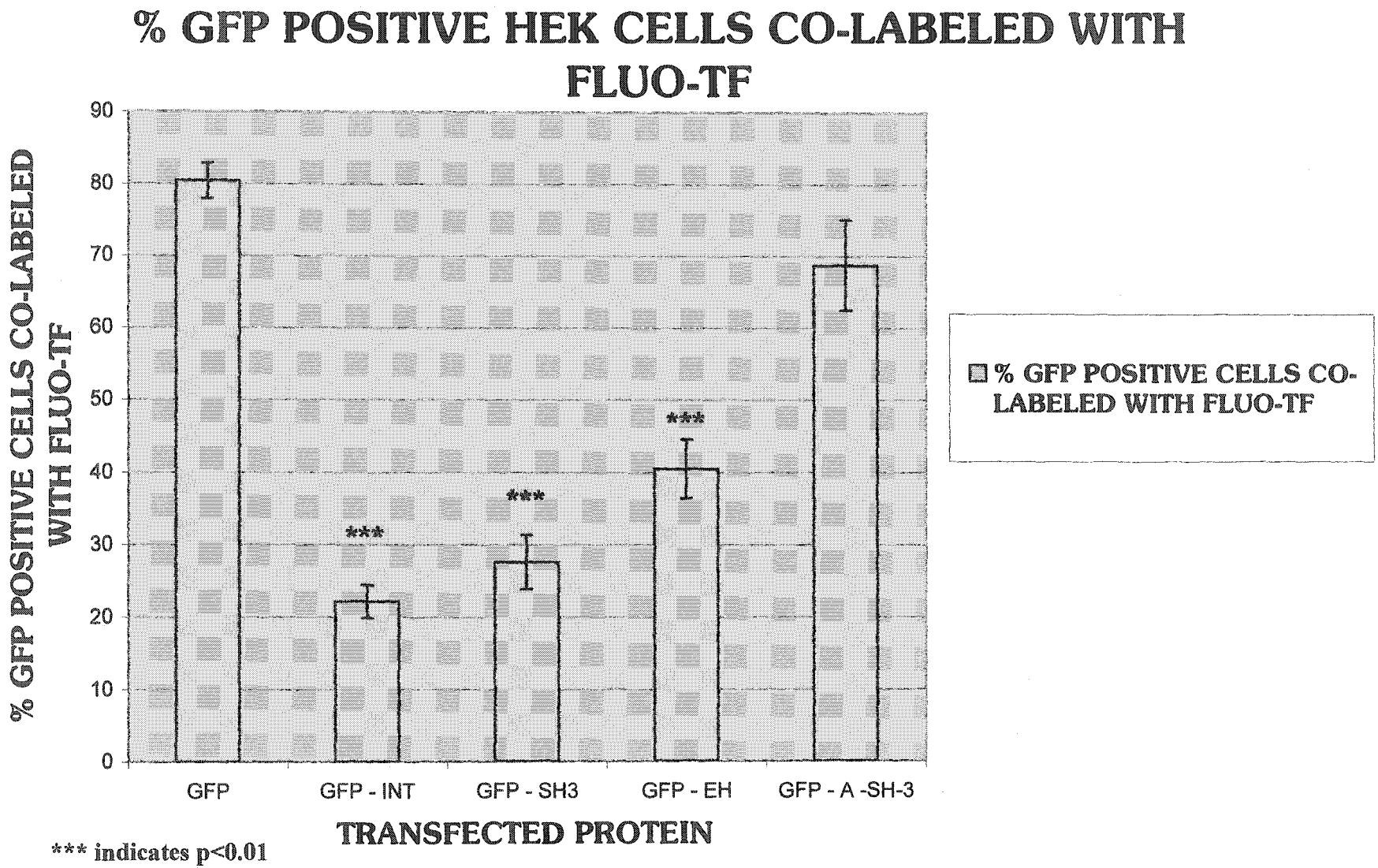


Figure 30 - Internalization of fluo-TF in HEK 293 cells in the presence of GFP or GFP-tagged constructs. The percentage of GFP positive cells that co-label with fluo-NT when HEK 293 cells are transfected with GFP is 80%. In HEK 293 cells transfected with GFP – INT, only 22% of GFP – INT positive cells co-labeled with fluo-TF, a value statistically significant from the value obtained when GFP alone is transfected into these cells ($p< 0.01$; $n=28$). In HEK 293 cells transfected with GFP - I - SH3, 28% of GFP - I - SH3 positive cells co-label with fluo-TF, a value that is again significantly different from that obtained in the control condition where GFP alone was transfected into HEK 293 cells ($p< 0.01$; $n=28$). HEK 293 cells transfected with GFP - I - EH demonstrated that 40% of GFP - I - EH positive cells co-labeled with fluo-TF, a value statistically different from the control value ($p<0.01$; $n=18$). The percentage of GFP – A – SH3 positive cells that co-labeled with fluo-TF was 69%, which was not statistically significant from the control value. These results indicate that intersectin, through its EH and SH3 domains, but not amphiphysin2, is implicated in fluo-TF internalization in HEK 293 cells.

FIGURE 30



*** indicates p<0.01

Table 3 – Summary of experimental results

Cell type	Construct	Affect on percentage of cells able to internalize fluo-NT	Affect on percentage of cells able to internalize fluo-TF
COS-7	BETA-ARRESTIN V53D	Decreased by 82%	Did not change
HEK 293	BETA-ARRESTIN V53D	Decreased by 67%	Did not change
COS-7	DYNAMIN K44A	Decreased by 78%	Decreased by 60%
HEK 293	DYNAMIN K44A	Decreased by 70%	Decreased by 47%
COS-7	GFP	Did not change	Did not change
HEK 293	GFP	Did not change	Did not change
COS-7	GFP-INT	Decreased by 58%	Decreased by 62%
HEK 293	GFP-INT	Did not change	Decreased by 65%
COS-7	GFP-I-SH3	Decreased by 65%	Decreased by 26%
HEK 293	GFP-I-SH3	Did not change	Decreased by 66%
COS-7	GFP-I-EH	Did not change	Did not change
HEK 293	GFP-I-EH	Did not change	Decreased by 50%
COS-7	GFP-A-SH3	Did not change	Did not change
HEK 293	GFP-A-SH3	Did not change	Did not change

Figure 31 – Proteins involved in the internalization of NTR1 in COS-7 and HEK 293 cells. COS-7 cells internalize fluo-NT in a time and temperature dependent manner which requires dynamin, beta-arrestin, and the SH3 domains of intersectin. HEK 293 cells internalize fluo-NT in a time and temperature dependent manner which requires dynamin and beta-arrestin.

Figure 31 - Proteins involved in the internalization of NTR1

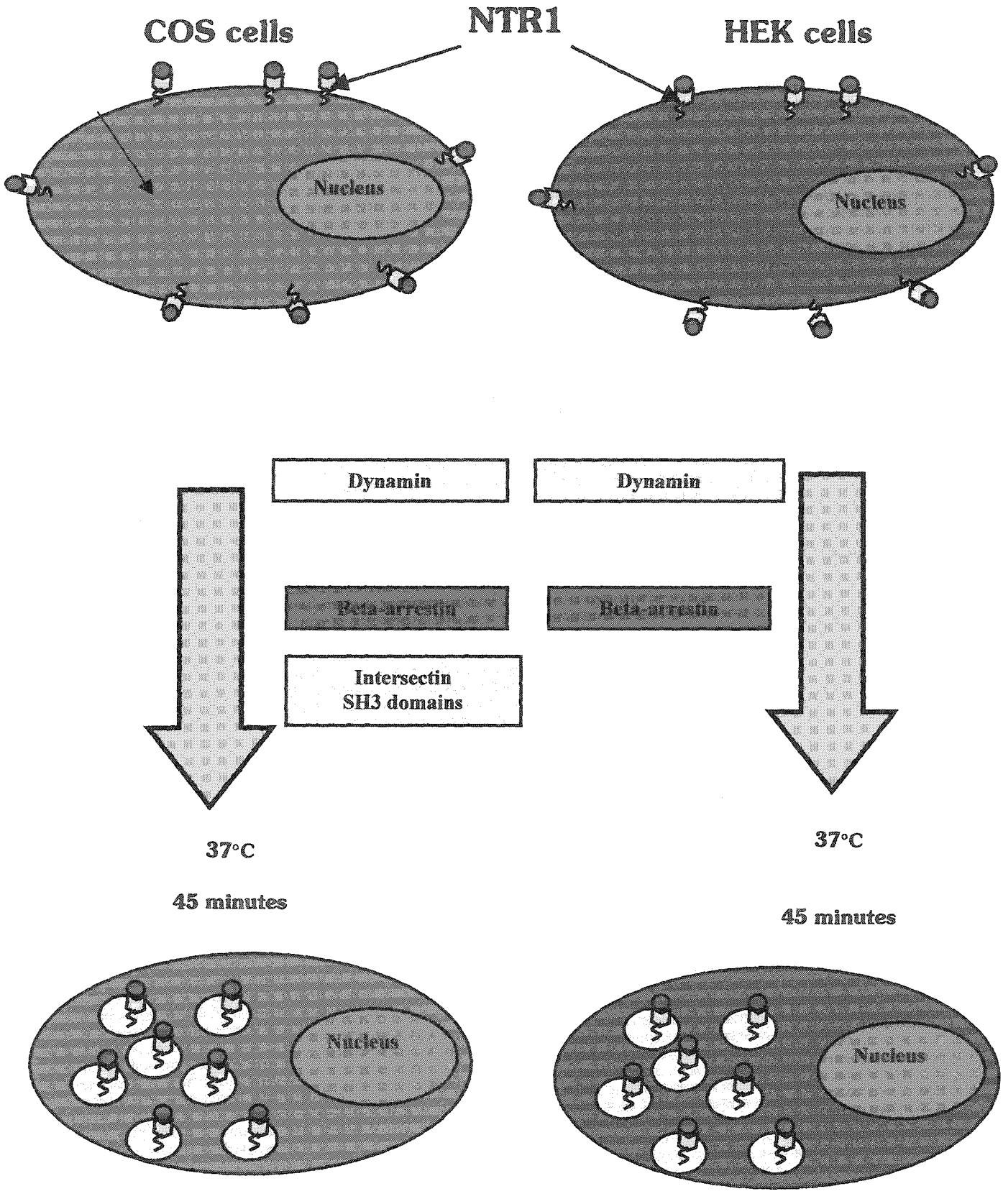
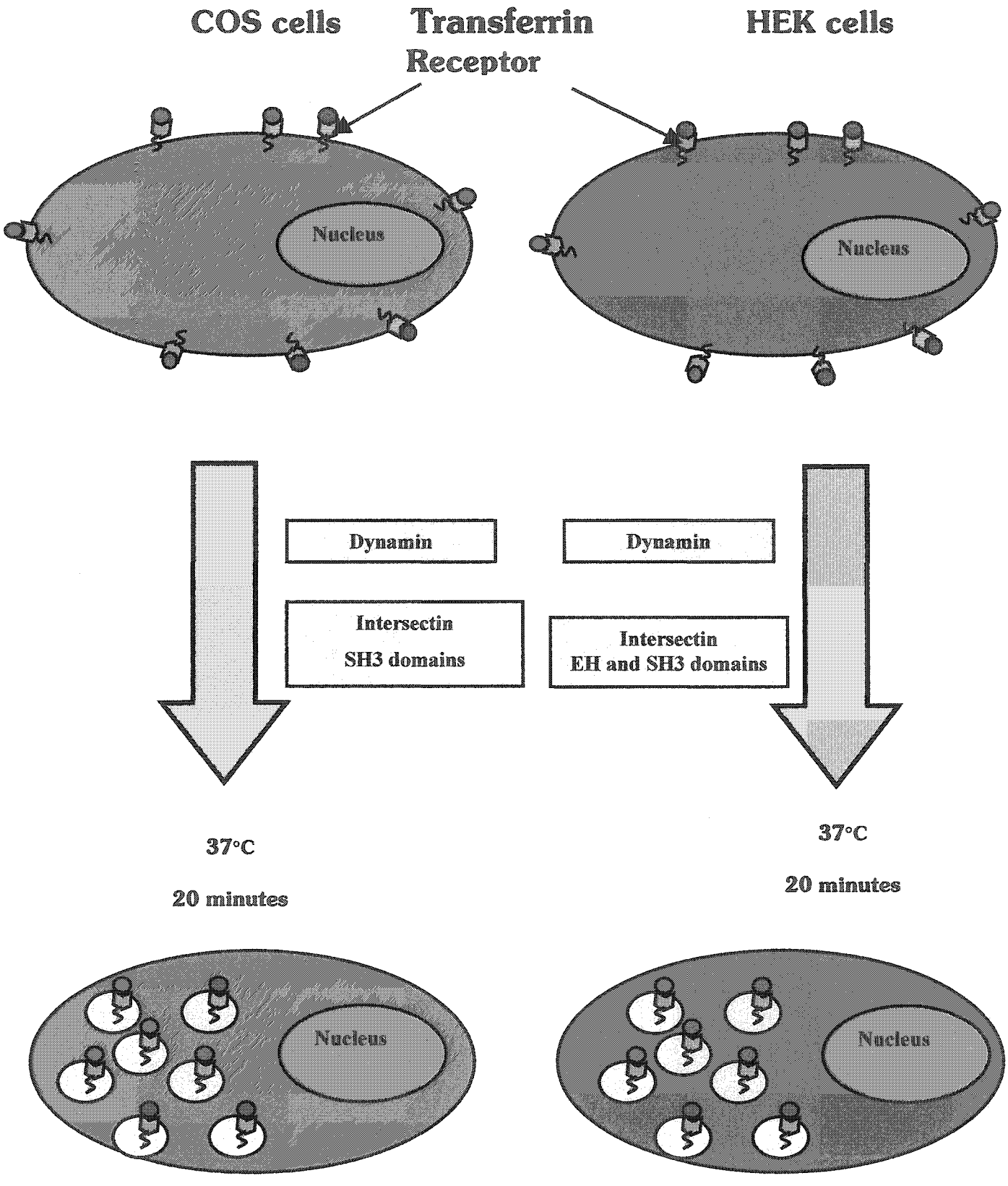


Figure 32 - Proteins involved in the internalization of the transferrin receptor in COS-7 and HEK 293 cells. COS-7 cells internalize fluo-TF in a time and temperature dependent manner which requires dynamin and the SH3 domains of intersectin. HEK 293 cells internalize fluo-NT in a time and temperature dependent manner which requires dynamin and the EH and SH3 domains of intersectin.

Figure 32 - Proteins involved in the internalization of fluo-TF



Chapter 4 - DISCUSSION

Endocytosis is an important process which allows all eukaryotic cells to interact with the extracellular environment by delivering nutrients, hormones, ions, transporters, and receptors into the cell. The internalization of receptors, and more specifically of GPCRs, is important for the initiation and regulation of intracellular signaling pathways. In addition, internalization of GPCRs following ligand binding allows for desensitization of the receptor and subsequent resensitization, thereby preventing continuous and uncontrolled activation. Thus, understanding the mechanisms that underlie GPCR internalization will allow us to gain critical insights into cellular functioning and homeostasis.

4.1 Internalization of NTR1 : A Model System

The internalization of NTR1 was chosen as a model system in which to study the internalization of GPCRs for a number of reasons. First, NTR1, a member of the GPCR superfamily, has been shown to internalize efficiently and reproducibly in a time, temperature, and agonist-mediated fashion in neurons as well as when expressed in mammalian cells. Some GPCRs, such as the SST1 receptor, internalize slowly and to a limited extent. Second, the cDNA of the cloned receptor was available, allowing for transfection into mammalian cell lines. Third, tools were available to permit the direct visualization of receptor internalization. Indeed, a fluorescent derivative of neurotensin, (fluo-NT) was also available, which had been demonstrated by Faure and colleagues in 1995 to bind with high affinity (Kd = 0.1 – 0.3 nM) to NTR1. This group also showed that upon stimulation with fluo-NT, the ligand and receptor enter the cell together in a time and temperature-dependent manner, indicating that fluo-NT could be used as a tool to assess NTR1 internalization.

4.2 NTR1 internalization

NTR1 has been shown to be internalized via a mechanism consistent with the involvement of clathrin-coated vesicles (Vandenbulke et al, 2000). Studies in COS-7 cells transfected with NTR1 have demonstrated that NTR1 co-localizes with its ligand upon entry into the cell (Vandenbulcke et al, 2000). The receptor/ligand complex dissociates in a step in the endocytic pathway subsequent to their clathrin-mediated internalization (Vandenbulcke et al, 2000). After 45 minutes, the ligand and NTR1 are fully dissociated. Whereas internalized receptors are targeted to lysosomes for degradation, the internalized ligand is recylced through the trans-golgi network (TGN) (Vandenbulcke et al, 2000).

A number of proteins have been implicated in the internalization of GPCRs, with beta-arrestin and dynamin figuring prominantly among them. Intersectin, a newly discovered protein (Yamabhai et al, 1998) binds to dynamin and has been shown to be involved in transferrin internalization. Amphiphysin2 has been shown to co-immunoprecipitate with synaptic vesicles, has a binding site for dynamin (David et al, 1996), and has also been proposed to be involved in transferrin internalization (Wigge et al, 1997).

The role of most of these proteins in the internalization of GPCRs, and more specifically for NTR1, has not been addressed. However, a recent study (Zhang et al, 1999) has shown that in HEK 293 cells transfected with NTR1, beta-arrestin translocates from the cytosol to the cell surface upon stimulation with NT, suggesting that beta-arrestin is involved in NTR1 internalization in this cell line. In addition, this neurotensin receptor co-localizes with beta-arrestin upon activation by agonist as observed by fluorescence microscopy. Although the effect of beta-arrestin on NTR1 internalization has been studied in HEK 293 cells, the results obtained in this cell system may not be extendable to other cellular systems.

Thus, to confirm the role of beta-arrestin in NTR1 internalization and to assess the involvement of dynamin, intersectin, and amphiphysin2 in this process, NTR1 internalization was examined in two different cell systems, HEK 293 and COS-7 cells. In parallel, these four proteins were assessed for their effects on the internalization of the transferrin receptor to differentiate cellular differences in endocytic machinery from differences between receptor types. The transferrin receptor is a single-transmembrane, constitutively internalized receptor which does not belong to the family of GPCRs, thereby allowing the determination of whether the same

endocytic machinery is used by the two classes of receptors. Thus the results of the current investigation are important, novel contributions to the study of GPCR internalization.

4.3 Methodological considerations

The availability of GFP-tagged intersectin and amphiphysin2 constructs as well as a fluorescent derivative of NT (fluo-NT) allowed for the use of a simple cell counting assay to quantitate NTR1 internalization. NTR1 internalization was assessed only in those cells that were positive for GFP or the GFP-tagged constructs, and NTR1. Internalization of NTR1 was determined by counting the percentage of total GFP or GFP-tagged contructs that were positive for fluo-NT binding, for both the TOTAL and ACID-WASH conditions. Using this method of analysis, the rates of transfection were not a limiting factor, since the method allowed us to ascertain that all the cells that were being quantitated contained the proteins of interest. In addition, fluorescently labeled cells would be analyzed by confocal microscopy, enabling one to gather further information on the trafficking of proteins inside the cell by sectioning cells at different depths.

On the other hand, due to the fact that mutant dynamin K44A and beta-arrestin V53D were not tagged to GFP, the effect of these mutants on the internalization of NTR1 was assessed by counting the percentage of total cells that were positive for fluo-NT in both the TOTAL and ACID-WASH conditions. This method of analysis is akin to experiments using radioligand binding or enzyme-linked assays to study receptor internalization, in that the entire population of cells is analyzed, and not only those that contain the two proteins of interest. It must be underscored that in transient transfections, only a certain percentage of cells are co-transfected with both proteins of interest. Thus, the largest drawback in analyzing internalization of a receptor in this manner is that the results obtained are a direct reflection of the extent of co-transfection of the two proteins of interest. Nonetheless, these methods of analyses have previously been used to determine that dynamin and beta-arrestin are involved in the internalization of many GPCRs, indicating that a substantial proportion of cells contain both plasmids. In the present study, although this method of analysis was not as precise as that used to study the effects of intersectin and amphiphysin2, the transfection efficiency of NTR1 in the presence of the K44A and V53D mutants was excellent, and a significant difference was noted between the percentage of cells that were fluo-NT positive in the TOTAL and ACID WASH

conditions, indicating that both mutant proteins, V53D and K44A, were effective in interfering with NTR1 internalization in both COS-7 and HEK 293 cells.

4.4 COS-7 and HEK 293 cells internalize fluo-NT and fluo-TF with different accumulation patterns and kinetics

Fluo-NT internalized in a time, temperature, and ligand-dependent manner in both COS-7 and HEK 293 cells transfected with the NTR1, although the labelling patterns of fluo-NT in the two cell types were not identical. After 45 minutes of incubation at 37°C, fluo-NT in COS-7 cells accumulated in a very distinct location in the cell, adjacent to the nucleus, while in HEK 293 cells, the labeling formed fluorescent "hot spots" distributed throughout the cytoplasm of the cell. A difference in labeling pattern between COS-7 and HEK 293 cells was also seen for fluo-transferrin (fluo-TF). COS-7 cells were clearly able to internalize fluo-TF more rapidly than HEK 293 cells, as the fluo-NT labeling was peri-nuclear in COS-7 cells after 20 minutes of incubation, but close to the plasma membrane in HEK 293 cells incubated with fluo-TF for the same time period. Thus, the time course of internalized ligand trafficking may differ between cell lines of internalization even in the absence of a dominant negative construct.

4.5 Beta-arrestin is involved in fluo-NT internalization in COS-7 and HEK 293 cells

The V53D beta-arrestin mutant, when overexpressed along with NTR1 in COS-7 cells, inhibited the internalization of fluo-NT, indicating that NTR1 internalizes via a beta-arrestin dependent pathway in this cell type. To date, no other studies have described the effects of beta-arrestin on NTR1 internalization in transfected COS-7 cells. However, many studies exist in the literature which detail the role of beta-arrestin in the internalization of many other GPCRs in COS-7 cells, such as the alpha (DeGraff et al, 1999) and beta-adrenergic receptors (Zhang et al, 1996), the angiotensin type 1A receptor (Gaborik et al, 2001), and the chemokine CXCR4 receptor (Orsini et al, 1999).

In HEK 293 cells co-transfected with NTR1 and V53D, the presence of the V53D mutant inhibited the internalization of fluo-NT. This finding concords with two recently published studies. Indeed, Zhang et al (1999) observed that stimulation of NTR1-transfected HEK 293 cells with NT mobilized GFP-tagged beta-arrestin molecules from the center of the cell to the plasma

membrane, and that NTR1 co-localized with beta-arrestin once internalized in the cell. These authors claim that this implies that NTR1 does not recycle, a finding that conforms to results obtained by Vandenbulcke et al in their 2000 publication. The second study, published by Oakley and colleagues in 2000, documented that NTR1, when transfected into HEK 293 cells, was able to directly bind to both beta-arrestin1 and beta-arrestin2. By contrast, other GPCRs have been shown to undergo a beta-arrestin independent internalization pathway, such as the B2-AR (Zhang et al,1996; Gagnon et al, 1998), the leutinizing hormone receptor (Lazari et al, 1998), m1, m2, m3, and m4 muscarinic receptors (Vogler et al, 1999), CXCR1 and CXCR4 chemokine receptors (Barlic et al, 1999; Orsini et al, 1999), and the AT1AR (Gaborik et al, 2001).

Curiously, it was observed that the transfection efficiency of NTR1 in the presence of beta-arrestin V53D was unusually high (double that for most of the other conditions). In addition, the labeling of fluo-NT in COS-7 cells co-transfected with the V53D beta-arrestin mutant was much more intense than HEK 293 cells co-transfected with the same construct. These two observations suggest an increase in cell surface receptors that could be the result of two possible scenarios. It is conceivable that in the presence of the beta-arrestin V53D plasmid, the expression of NTR1 may be upregulated in some way, producing more NTR1 protein. Indeed, it has recently been shown that beta-arrestin plays a role in signal transduction (Miller et al, 2001a,b; Miller et al, 2001). In fact, beta-arrestins have been shown to contribute to the assembly of signaling protein complexes. For example, evidence that indicates that beta-arrestin plays a role in signaling via c-Src tyrosine kinases comes from a study examining the role of tyrosine kinase activation in the degranulation of neutrophils. CXCR1-mediated granule release and tyrosine kinase activation were inhibited in the presence of a dominant negative mutant beta-arrestin (P91G-P121E) that is unable to bind to c-Src kinase (Luttrell et al, 1999; Baric et al, 2000). Beta-arrestins have also been shown to interact directly with Raf-1 kinase and MAP kinase (DeFea et al, 2000). Thus, the mutant protein may possess the ability to recruit more NTR1 to the plasma membrane by modulating a signaling cascade.

Furthermore, it has been shown that the stimulation of NTR1 induces the MAP kinase pathway both in cells in which it is endogenously expressed (HT-29 cells), as well as in CHO cells in which the NTR1 was transiently expressed (Poinot-Chazel et al, 1996). It is therefore conceivable that beta-arrestin forms a part of the NTR1/MAP kinase signaling complex. If a functional beta-arrestin is absent, as would be in the case of cells transfected with the beta-arrestin V53D mutant, MAP kinase signaling normally induced by NTR1 may be perturbed and may

account for the apparent increase in recruitment of NTR1 to the cell surface seen in COS-7 cells co-transfected with NTR1 and beta-arrestin V53D.

Transcriptional effects of neurotensin on mRNA synthesis of NTR1 in NIE-115 cells was determined to be dependent on proper internalization of the NT/NTR1 complex, as these effects were blocked in the presence of PAO or concanavalin A, agents that inhibit endocytosis (Souaze et al, 1997). Hence, another possibility to explain the altered recruitment of NTR1 could be due to the inhibition of neurotensin's transcriptional effects due to inefficient NT/NTR1 internalization caused by the mutant V53D beta-arrestin.

Nonetheless, the increase in fluo-NT binding observed was exclusive to COS-7 cells co-transfected with the beta-arrestin V53D plasmid and NTR1, suggesting that the same molecule could have different actions depending on which cell type it is expressed.

The mutant V53D had no effect on the internalization of fluo-TF when overexpressed in either COS-7 or HEK 293 cells. This result confirms the belief that the role of beta-arrestin in receptor endocytosis may be limited to GPCRs. Indeed, beta-arrestin has a strong affinity for phosphorylated GPCRs, but not other types of receptors (Lohse et al, 1992).

4.6 Dynamin is involved in fluo-NT internalization in both COS-7 and HEK 293 cells

The mutant dynamin K44A, when overexpressed in both COS-7 and HEK 293 cells along with NTR1, proved a potent inhibitor of fluo-NT (and hence of NTR1) internalization. Many other GPCRs have been shown to internalize via a dynamin-dependent pathway in COS-7 cells, such as the B2-AR (Zhang et al, 1996; Gagnon et al, 1998), AT1AR (Gaborik et al, 2001), muscarinic m1, m2, m3, and m4 receptors (Vogler et al, 1998), bradykinin B2 receptor (Pizard et al, 1999), and the CXCR4 chemokine receptor (Orsini et al, 1998). GPCRs known to internalize via a dynamin-dependent pathway in HEK 293 cells include the B2-AR, (Zhang et al, 1996; Gagnon et al, 1998), AT1AR (Gaborik et al, 2001), LH receptor (Lazari et al, 1998), dopamine D1 receptor (Vickery et al, 1999), the bradykinin B2 receptor (Pizard et al, 1999), the CXCR4 chemokine receptor (Orsini et al, 1999), m2 muscarinic receptor (Werbonat et al, 2000), and 5-HT2A receptor (Bhatnagar et al, 2001). However, the present study is the first to report a role for dynamin in NTR1 internalization in either cell type. Fluo-TF internalization was also inhibited by

the mutant dynamin in both COS-7 and HEK 293 cells, in concordance with previous studies that have linked dynamin to the internalization of the transferrin receptor in these cell lines (Vallis et al, 1999; Barlic et al, 1999).

4.7 The role of intersectin in NTR1 internalization

4.7.1 Full-length Intersectin

Overexpression of full length intersectin was found to be a potent inhibitor of fluo-NT internalization in COS-7 cells co-transfected with NTR1. This result was rather surprising, since overexpression of other proteins involved in the endocytic machinery, such as beta-arrestin, was previously found to enhance GPCR internalization, presumably to its maximal capacity, or to be without effect, if the protein is not a rate-limiting factor of internalization in that cell type (Vogler et al, 1999). The ability of intersectin to inhibit NTR1 internalization may be due to the fact that it is composed of multiple domains, including EH and SH3 domains, enabling it to bind to many other molecules. Indeed, the SH3 domains of intersectin are capable of binding to proline-rich molecules, such as dynamin, while its EH domains are capable of binding to NPF containing proteins, such as Eps15, which in turn can bind to clathrin. Thus, it is possible that overexpression of intersectin may sequester proteins of the endocytic machinery in the cytosol and prevent them from reaching the plasma memebrane. Alternatively, since the middle portion of intersectin mediates its binding to other intersectin molecules, it is possible that an increased level of intersectin would mediate augmented intersectin-intersectin interactions, thus sterically hindering intersectin from being efficiently targeted to the plasma membrane, and preventing it from binding efficiently to dynamin or Eps15. The hypothesis that overexpression of full length intersectin prevents proper targeting of the protein to the plasma membrane is strengthened by the observation that full-length GFP-tagged intersectin adopts a punctate distribution in COS-7 cells when transfected in this cell line. Thus, intersectin appears to be sequestered in intracellular compartments, suggesting that the endocytic machinery are not targeted to the plasma membrane where an abundance of clathrin-coated pits are found.

Overexpression of full-length intersectin also inhibited the internalization of the transferrin receptor in COS-7 cells, presumably through similar mechanisms, as the same punctate distribution of intersectin was observed in COS-7 cells transfected with intersectin.

By contrast, overexpression of full length intersectin did not disrupt the internalization of fluo-NT in HEK 293 cells, even though it adopted the same globular, punctate distribution as seen in COS-7 cells. Contrastingly, fluo-TF internalization in HEK 293 cells was potently inhibited by intersectin overexpression, suggesting that intersectin is endogenously expressed in this cell line. These results thus suggest that this form of intersectin is not involved in the internalization of NTR1 in HEK 293 cells, even though it is involved in the internalization of the transferrin receptor in this cell line. The punctate distribution pattern lends further support to the sequestration hypothesis. Nonetheless, since intersectin is a relatively newly discovered protein, the possibility remains that another as yet unidentified form or subtype of intersectin may exist in this cell line that is specific to the internalization of certain other classes of receptors, such as GPCRs.

The observation that intersectin is involved in fluo-NT internalization in COS-7 cells co-transfected with NTR1 but not in HEK 293 cells co-transfected with GFP-tagged intersectin is an interesting, although not uncommon, finding. It is well established in cell biology literature that different receptors internalize via different mechanisms or to different extents depending on the cell type in which they are expressed. For example, CXCR1, which is the receptor for the chemokine interleukin 8 (IL-8), does not internalize in HEK 293 cells, but does in RBL-2H3 cells (rat basophilic leukemia cells) (Barlic et al, 1999). The muscarinic receptor, subtype 1 (m1) internalizes slowly in JEG-3 cells (Goldman et al, 1996) but rapidly in Y1 and HEK 293 cells (Scherer et al, 1990: Lee et al, 1998). The delta opioid receptor has been shown to undergo phosphorylation-dependent internalization in CHO cells, but phosphorylation independent internalization in HEK 293 cells (Murray et al, 1998). In COS-7 cells, B2-AR internalizes poorly (10%), but in HEK 293 cells, B2-AR internalizes robustly (55%) (Menard et al, 1997). Finally, B2-AR internalize via caveolae in A431 cells, but not in COS-1, HEK 293, or HeLa cells (Gagnon et al, 1998).

4.7.2 The SH3 domains of Intersectin

In COS-7 cells transfected with NTR1, overexpression of the SH3 domains of intersectin was able to inhibit fluo-NT internalization, suggesting that these domains are important for the internalization of NTR1 in this cell line. It has been shown in COS-7 cells that intersectin contains five SH3 domains per molecule (Yamabhai et al, 1998). The SH3 A, B and C domains of intersectin bind dynamin with high affinity, while intersectin's SH3 D and E domains bind dynamin with lower affinity. Therefore, for every string of SH3 domains of intersectin transfected into the cell, 3-5 endogenous dynamin molecules would potentially be sequestered. As a result, even if dynamin was expressed in large amounts in COS-7 cells, most of it would bind to the overexpressed SH3 domains of intersectin, thereby preventing it from binding to endogenous full-length intersectin. The fact that stimulation with fluo-NT did not target GFP-I-SH3 to the plasma membrane in COS-7 cells suggests that the SH3 domain is not one which is involved in targeting intersectin to the plasma membrane. Since the SH3 domains alone are unable to target the complex to the site of coated pits, dynamin and intersectin's other SH3-domain binding partners would be sequestered with the SH3 domains intracellularly. In fact, the GFP-I-SH3 labeling was seen throughout the cell, supporting this hypothesis. Hence, overexpression of GFP-I-SH3 domains may prevent NTR1 internalization by trapping endogenous dynamin in the cytosol and failing to target it to the plasma membrane to the sites of clathrin-coated pits. The SH3 domains of intersectin, when overexpressed in COS-7 cells, also inhibited fluo-TF internalization in COS-7 cells, presumably through a similar mechanism.

In contrast, internalization of fluo-NT was not inhibited in HEK 293 cells co-transfected with NTR1 and GFP-I-SH3. This finding is an important one, and concords with the findings from the experiments with full-length intersectin that this protein is not involved in HEK 293 cells. By contrast, fluo-TF internalization was severely affected in HEK 293 cells overexpressing GFP-I-SH3. These results suggest that the SH3 domain is necessary for the internalization of the transferrin receptor in HEK 293 cells. Thus these results confirm that intersectin is not only expressed in this cell line but that it plays a role in the internalization of select receptors.

4.7.3 The EH Domains of intersectin

COS-7 and HEK 293 cells co-transfected with NTR1 and GFP-I-EH internalized fluo-NT normally, suggesting that the EH domains of intersectin are not involved in the internalization of

NTR1. Eps15, which is a protein similar to intersectin in that it contains multiple EH domains, has been shown to localize to the rim of clathrin-coated pits. Furthermore, it has been shown that EH domains are responsible for targeting Eps15 to the rim of coated pits. Perhaps COS-7 and HEK 293 cells use Eps 15 as their source of EH domains, which, when bound to other proteins that are part of the endocytic machinery, target the complex to clathrin-coated pits. As a result, overexpression of the EH domains of intersectin would not interfere with internalization of fluo-NT, as the EH domains in Eps 15 may have a higher affinity for clathrin-coated pits and cytosolic endocytic machinery than the EH domans of intersectin.

COS-7 cells were likewise not inhibited from internalizing fluo-TF when the EH domains of intersectin were overexpressed in this cell line. Interestingly, however, HEK 293 cells were inhibited from internalizing fluo-TF when the EH domains of intersectin were overexpressed. These results clearly illustrate that the mechanism of endocytosis used to internalize transferrin differ, as do the mechanisms of endocytosis used to internalize NTR1, in COS-7 and HEK 293 cells. COS-7 cells require the SH3 domains of intersectin, but not its EH domains to internalize the transferrin receptor. HEK 293 cells require both the SH3 and EH domains of intersectin to internalize the transferrin receptor. These results further support the notion that receptor internalization mechanisms are not universal between different cell types, or even within the same cell, as, in the present work, it was found that two different receptors (NTR1 and the transferrin receptor) employed different internalization machinery in the same cell line (HEK 293 cells).

4.7.4 Concluding Remarks on the role of intersectin in NTR1 internalization

COS-7 cells have been previously reported to express endogenous intersectin (Yamabhai et al, 1999). The results of the present study suggest that HEK 293 cells also contain endogenous intersectin, since fluo-TF internalization was affected by overexpression of intersectin or its SH3 and EH domains. Endogenous levels of intersectin in these two cell types may be relevant in the analysis of the present study. Indeed, high endogenous levels of intersectin in HEK 293 cells might result in inefficient competition of endogenous intersectin with overexpressed constructs, leading to the observed lack of effect of intersectin on NTR1 internalization in this cell line. On the other hand, HEK 293 cells may contain less endogenous intersectin than COS-7 cells. In this scenario, fluo-NT internalization, which may normally not use an intersectin-dependent internalization pathway in HEK 293 cells, may be recruited to one when an overexpression of

intersectin is introduced into the cell. In this case, internalization of fluo-NT would still be observed. Overexpression of intersectin's EH or SH3 domains in HEK 293 cells would have no effect on fluo-NT internalization, as the cell would use the alternate, intersectin-independent pathway when insufficient levels of endogenous intersectin are present in the cell.

It is well-established that the levels of proteins involved in receptor-mediated endocytosis differ according to cell type, and thus affect the internalization pathway a cell employs to internalize a particular receptor. For example, lung microvascular epithelium cells have very little clathrin-coated pits at the plasma membrane, but a high level of caveolae (Oh et al, 1998). Liver endothelial cells contain many clathrin-coated pits, but no caveolae (Oh et al, 1998). COS-7 cells possess caveolae but HeLA cells do not (Oh et al, 1998). COS-7 cells contain 70% less beta-arrestin and GRK than HEK 293 cells (Menard et al, 1997). HEK 293 cells have a lower expression of beta-arrestins than RBL-2H3 cells (Barlic et al, 1999). Furthermore, endothelial cells change phenotypically when isolated and grown in culture; they have both clathrin coated pits and caveolae on their cell surface although the density of caveolae is at least 10 fold less *in vitro* than *in vivo* (Schnitzer, 1996). In addition, the affinities the endocytic proteins have for the particular receptor in a specific cell line will affect the internalization mechanism used by a cell to internalize that particular receptor. Finally, factors such as the architecture of the cell and total protein levels may affect interactions between the endocytic machinery and a receptor by creating steric or physical hindrances and weaken (or strengthen) these important protein-protein interactions.

4.8 Amphiphysin2 is not involved in fluo-NT internalization in either COS-7 or HEK 293 cells

GFP-A-SH3, the GFP-tagged SH3 domain of amphiphysin2, failed to interfere with fluo-NT and fluo-TF internalization in either COS-7 or HEK 293 cells. The technique employed in this work has the advantage of allowing for direct visualization of the phenomenon being measured and hence provide convincing evidence that fluo-TF and fluo-NT internalization is not inhibited in the presence of an overexpression of the SH3 domain of amphiphsyin2. Nonetheless, these results do not preclude an amphiphysin-dependent internalization mechanism for fluo-NT and fluo-TF in COS-7 and HEK 293 cells. Indeed, if both COS-7 and HEK 293 cells express a large amount of dynamin, a sufficient quantity might be able to bind to endogenous amphiphysin2, or to endogenous intersectin (which contains five SH3-binding sites), allowing

internalization to occur successfully. Alternatively, another form of amphiphysin could be present in COS-7 and HEK 293 cells, whose SH3 domain binds dynamin with higher affinity than the SH3 domain of amphiphysin2, the isoform used in this study.

Although the role of amphiphysin in the internalization of the transferrin receptor has been studied at length, the effect of amphiphysin2 on the internalization of a GPCR had not been examined prior to the present work. The majority of studies published on this topic have proposed that the injection or transfection of the SH3 domain of amphiphysin into cells inhibits transferrin internalization. However, a study by Grabs et al in 1997 showed that transfection into COS-7 cells of dynamin with a mutated amphiphysin binding site (PSRPNR to PSRPNE) did not affect transferrin receptor internalization. Furthermore, Sarret et al in 2001 have indicated that stable transfection in Att-20 cells of amphiphysin 2b, an amphiphysin isoform which lacks one of the two putative clathrin binding domains, failed to inhibit the internalization of the somatostatin receptors SST2A and SST5, members of the GPCR superfamily. The amphiphysin 2b isoform used in the study by Sarret contains only one clathrin binding site, and thus should exhibit a reduced affinity for clathrin-coated pits compared to the wild-type amphiphysin2. It would therefore be expected that the internalization of the somatostatin receptors would be inhibited at least partially. The results obtained in the present study provide evidence that amphiphysin2 has a limited role in the internalization of another GPCR, the NTR1. The results also are in concordance with the study by Grabs and colleagues discussed above, in which transferrin internalization was not inhibited despite the presence of a mutant dynamin that could not bind to amphiphysin. Finally, three recent studies in Drosophila which lack all amphiphysin function (i.e. are null for the amphiphysin gene) suggest that synaptic vesicle endocytosis is not perturbed in these mutant flies (Leventis et al, 2001; Zelhof et al, 2001; Razzaq et al, 2001).

4.9 Future studies

The level of agonist to which a receptor is exposed appears to influence the involvement of particular proteins in the internalization of a receptor. In a 2001 study by Gaborik et al., COS-7, HEK 293, and CHO cells were shown to internalize AT1AR in an arrestin and dynamin-dependent way, as the V53D mutant and a clathrin-binding mutant of arrestin, as well as the dynamin2 mutants K44A and K535A (impaired in phospholipid binding), inhibited internalization of AT1AR in all three cell types. However, this dependence was dependent on the concentration of ligand the cells were stimulated with. At higher agonist concentrations, inhibitors lost their

effect. Therefore, at physiological concentrations of angiotensin, AT1AR internalizes via a beta-arrestin and dynamin-dependent way (Gaborik et al, 2001). Consequently, it would be very interesting to repeat the experiments described in the present study using different concentrations of fluo-NT and to ascertain whether the endocytic machinery used to internalize NTR1 in both COS-7 and HEK 293 cells was altered. Perhaps at a certain concentration of agonist, a dependence on intersectin of NTR1 internalization in HEK 293 cells co-transfected with NTR1 and GFP-tagged intersectin constructs would be seen that was not apparent at an agonist concentration of 20nM.

Although not specifically addressed in this study, it would also be of interest to determine whether or not NTR1 internalizes in part by a caveolae-mediated pathway, as do some GPCRs in certain cell types (i.e. CCK receptors in CHO cells (Roettger et al, 1995), M2 muscarinic receptors in cardiac myocytes (Feron et al, 1997), B2-AR in human epidermoid carcinoma A-431 cells (Dupree et al, 1993), and bradykinin (de Weerd et al, 1997). To determine whether or not NTR1 internalizes via caveolae in COS-7 or HEK 293 cells, a number of experimental approaches could be used. One is to perform electron microscopy (EM) on COS-7 or HEK 293 cells that have been transfected with NTR1 and stimulated with neurotensin after varying periods of time to determine if caveolae could be seen to be directly associated with NTR1. Receptor internalization via clathrin-coated pit formation can be disrupted by specific treatments that will not disrupt caveolae-mediated internalization. For example, potassium depletion substantially decreases clathrin-mediated internalization while not interfering with caveolae mediated internalization of the CCK receptor (Roetgger et al, 1995). Conversely, there are certain treatments that do not disrupt clathrin-mediated internalization but do disrupt caveolae-mediated internalization. An example would be sterol-binding drugs, such as filipin, nystatin, and digitoxin (Schnitzer et al, 1994). Therefore, a second approach to determine the whether there is caveolae-mediated internalization of NTR1 in COS-7 or HEK 293 cells would be to incubate the transfected, stimulated cells with one of these three agents to determine if internalization of NTR1 is decreased from baseline levels. Given that the results in this study suggest that different cell types do not necessarily use the same proteins to internalize receptors, it would be of interest to determine whether COS-7 and HEK 293, use caveolae-mediated internalization to the same extent (if any), to internalize NTR1.

As all the work in the present study was done in mammalian cell lines, there is still the need for studies of NTR1 internalization on native tissues or primary isolates of cells that

endogenously express NTR1. The results of the present study, however, can be used to develop powerful tools for determining the necessary requirements for NTR1 internalization in vivo. The constructs used in this study can be microinjected, transfected, or infected (in the case of adenovirus gene delivery) into neurons themselves, then exposed to fluo-NT and subsequently monitored for NTR1 internalization.

4.10 Implication of experimental results

4.10.1 NTR1 and disease

The present work concentrated on the study of the high affinity neurotensin receptor (NTR1). Many of the central effects of neurotensin on dopaminergic transmission are mediated through its interaction with the NTR1. Indeed, neurotensin and NTR1 co-localize with dopamine in many areas of the brain (Mai, 1987; Quirion, 1983; Nemeroff, 1986; Vincent et al, 1999; Kinkead et al, 1999). The dopaminergic system is altered in schizophrenia, and it was subsequently found that neurotensin function in schizophrenics is affected as well. Lower levels of NT are found in the CSF of schizophrenics (Sharma et al, 1997; Garver et al, 1991), and the brain of schizophrenics display a decreased amount of NTR1 (Wolf et al, 1995; Lahti et al, 1998). Thus, NTR1 has been implicated in the pathophysiology of schizophrenia, and a specific antagonist of NTR1, SR 48692, is currently in stage III clinical trials as an anti-psychotic to treat this disease (A. Beaudet, personal communication).

The results of the present experiments might enable a drug to be developed based on one of the constructs that inhibit NTR1 internalization in vivo, and thereby modulate neuronal responsiveness to NT. It has been shown that NTR1 levels are underexpressed in schizophrenics (Wolf et al, 1995; Lahti et al, 1998). Thus, enabling more NTR1 to remain on the surface of neurons for a longer period of time, by inhibiting their internalization, might have a beneficial effect on the modulation of dopamine transmission in terms of alleviating schizophrenic symptoms.

At the periphery, cancerous lung, thyroid, and prostate cells have all been shown to contain and secrete neurotensin (Kapuscinski et al, 1990; Moody et al, 1985; Seghal et al, 1994; Wood et al, 1981; Zeytinoglu et al, 1980). Incubation of lung cancer cells with NT increases their

number by 2.5 times, an effect that can be inhibited with the NTR1 specific antagonist SR 48692 (Moody et al, 2001). These findings suggest that the effect of NT on cell growth and carcinogenesis is mediated at least in part by the effects of NT on NTR1. Therefore, certain prostate, lung, and thyroid tumors which overexpress NTR1 may be amenable to radiotherapy. In fact, tumors that overexpress somatostatin receptors are currently being treated with radiolabeled somatostatin (Koenig et al, 1998). Hence the principles behind receptor-mediated endocytosis can be applied clinically to the therapy of some forms of cancer. Additionally, as NT stimulates cancerous growth of certain cells through binding and internalizing NTR1, inhibiting agonist-mediated NTR1 internalization by using the endocytosis inhibitors discovered in this work would decrease NT signaling, as well as its deleterious effects.

Conclusions

- NTR1 uses a dynamin- and beta-arrestin-dependent pathway in COS-7 cells and HEK 293 cells; NTR1 internalizes in an intersectin-dependent manner in COS-7 but not HEK 293 cells.
- Transferrin uses an intersectin- and dynamin-dependent internalization pathway in both COS-7 and HEK 293 cells.
- NTR1 and the tranferrin receptor internalize in an amphiphysin2 independent pathway in both COS-7 and HEK 293 cells.
- Beta-arrestin is not involved in the internalization of the transferrin receptor in either COS-7 or HEK 293 cells.

REFERENCES

Achiriloaie, M., Barylko, B. & Albanesi, J. P. (1999). Essential role of the dynamin pleckstrin homology domain in receptor- mediated endocytosis. *Mol Cell Biol* **19**, 1410-5.

al-Rodhan, N. R., Richelson, E., Gilbert, J. A., McCormick, D. J., Kanba, K. S., Pfenning, M. A., Nelson, A., Larson, E. W. & Yaksh, T. L. (1991). Structure-antinociceptive activity of neurotensin and some novel analogues in the periaqueductal gray region of the brainstem. *Brain Res* **557**, 227-35.

Amar, S., Kitabgi, P. & Vincent, J. P. (1986). Activation of phosphatidylinositol turnover by neurotensin receptors in the human colonic adenocarcinoma cell line HT29. *FEBS Lett* **201**, 31-6.

Anborgh, P. H., Seachrist, J. L., Dale, L. B. & Ferguson, S. S. (2000). Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors. *Mol Endocrinol* **14**, 2040-53.

Arriza, J. L., Dawson, T. M., Simerly, R. B., Martin, L. J., Caron, M. G., Snyder, S. H. & Lefkowitz, R. J. (1992). The G-protein-coupled receptor kinases beta ARK1 and beta ARK2 are widely distributed at synapses in rat brain. *J Neurosci* **12**, 4045-55.

Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G. & Lefkowitz, R. J. (1992). Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J Biol Chem* **267**, 17882-90.

Baba, T., Damke, H., Hinshaw, J. E., Ikeda, K., Schmid, S. L. & Warnock, D. E. (1995). Role of dynamin in clathrin-coated vesicle formation. *Cold Spring Harb Symp Quant Biol* **60**, 235-42.

Barak, L. S., Oakley, R. H., Laporte, S. A. & Caron, M. G. (2001). Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. *Proc Natl Acad Sci U S A* **98**, 93-8.

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. & Caron, M. G. (1994). A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *J Biol Chem* **269**, 2790-5.

Barlic, J., Andrews, J. D., Kelvin, A. A., Bosinger, S. E., DeVries, M. E., Xu, L., Dobransky, T., Feldman, R. D., Ferguson, S. S. & Kelvin, D. J. (2000). Regulation of tyrosine kinase activation and granule release through beta-arrestin by CXCRI. *Nat Immunol* 1, 227-33.

Barlic, J., Khandaker, M. H., Mahon, E., Andrews, J., DeVries, M. E., Mitchell, G. B., Rahimpour, R., Tan, C. M., Ferguson, S. S. & Kelvin, D. J. (1999). beta-arrestins regulate interleukin-8-induced CXCR1 internalization. *J Biol Chem* **274**, 16287-94.

Beaudet, A., Mazella, J., Nouel, D., Chabry, J., Castel, M. N., Laduron, P., Kitabgi, P. & Faure, M. P. (1994). Internalization and intracellular mobilization of neurotensin in neuronal cells. *Biochem Pharmacol* **47**, 43-52.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N. & Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci* **112**, 1303-11.

Benmerah, A., Begue, B., Dautry-Varsat, A. & Cerf-Bensussan, N. (1996). The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein. *J Biol Chem* **271**, 12111-6.

Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G. & Lefkowitz, R. J. (1987). Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc Natl Acad Sci U S A* **84**, 8879-82.

Berry, S. A., Shah, M. C., Khan, N. & Roth, B. L. (1996). Rapid agonist-induced internalization of the 5-hydroxytryptamine2A receptor occurs via the endosome pathway in vitro. *Mol Pharmacol* **50**, 306-13.

Bhatnagar, A., Willins, D. L., Gray, J. A., Woods, J., Benovic, J. L. & Roth, B. L. (2001). The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT2A) serotonin receptors reveals differential sorting of arrestins and 5-HT2A receptors during endocytosis. *J Biol Chem* **276**, 8269-77.

Botto, J. M., Chabry, J., Sarret, P., Vincent, J. P. & Mazella, J. (1998). Stable expression of the mouse levocabastine-sensitive neurotensin receptor in HEK 293 cell line: binding properties, photoaffinity labeling, and internalization mechanism. *Biochem Biophys Res Commun* **243**, 585-90.

Boudin, H., Sarret, P., Mazella, J., Schonbrunn, A. & Beaudet, A. (2000). Somatostatin-induced regulation of SST(2A) receptor expression and cellsurface availability in central neurons: role of receptor internalization. *J Neurosci* **20**, 5932-9.

Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G. & Lefkowitz, R. J. (1988). Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature* **333**, 370-3.

Bradbury, F. A. & Menon, K. M. (1999). Evidence that constitutively active luteinizing hormone/human chorionic gonadotropin receptors are rapidly internalized. *Biochemistry* **38**, 8703-12.

Brand, S. H., Laurie, S. M., Mixon, M. B. & Castle, J. D. (1991). Secretory carrier membrane proteins 31-35 define a common protein composition among secretory carrier membranes. *J Biol Chem* **266**, 18949-57.

Bremnes, T., Paasche, J. D., Mehlum, A., Sandberg, C., Bremnes, B. & Attramadal, H. (2000). Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* **275**, 17596-604.

Brodsky, F. M., Hill, B. L., Acton, S. L., Nathke, I., Wong, D. H., Ponnambalam, S. & Parham, P. (1991). Clathrin light chains: arrays of protein motifs that regulate coated- vesicle dynamics. *Trends Biochem Sci* **16**, 208-13.

Bundgaard, M. (1991). The three-dimensional organization of smooth endoplasmic reticulum in capillary endothelia: its possible role in regulation of free cytosolic calcium. *J Struct Biol* **107**, 76-85.

Buss, F., Arden, S. D., Lindsay, M., Luzio, J. P. & Kendrick-Jones, J. (2001). Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis. *Embo J* **20**, 3676-84.

Butler, M. H., David, C., Ochoa, G. C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O. & De Camilli, P. (1997). Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/Rvs family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of ranvier in brain and around T tubules in skeletal muscle. *J Cell Biol* **137**, 1355-67.

Cao, H., Garcia, F. & McNiven, M. A. (1998). Differential distribution of dynamin isoforms in mammalian cells. *Mol Biol Cell* **9**, 2595-609.

Carraway, R., Cochrane, D. E., Lansman, J. B., Leeman, S. E., Paterson, B. M. & Welch, H. J. (1982). Neurotensin stimulates exocytotic histamine secretion from rat mast cells and elevates plasma histamine levels. *J Physiol* **323**, 403-14.

Carraway, R. & Leeman, S. E. (1973). The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J Biol Chem* **248**, 6854-61.

Carroll, R. C., Beattie, E. C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R. A., Malenka, R. C. & von Zastrow, M. (1999). Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc Natl Acad Sci U S A* **96**, 14112-7.

Castel, M. N., Malgouris, C., Blanchard, J. C. & Laduron, P. M. (1990). Retrograde axonal transport of neurotensin in the dopaminergic nigrostriatal pathway in the rat. *Neuroscience* **36**, 425-30.

Chabry, J., Botto, J. M., Nouel, D., Beaudet, A., Vincent, J. P. & Mazella, J. (1995). Thr-422 and Tyr-424 residues in the carboxyl terminus are critical for the internalization of the rat neurotensin receptor. *J Biol Chem* **270**, 2439-42.

Chabry, J., Labbe-Jullie, C., Gully, D., Kitabgi, P., Vincent, J. P. & Mazella, J. (1994). Stable expression of the cloned rat brain neurotensin receptor into fibroblasts: binding properties, photoaffinity labeling, transduction mechanisms, and internalization. *J Neurochem* **63**, 19-27.

Chalon, P., Vita, N., Kaghad, M., Guillemot, M., Bonnin, J., Delpech, B., Le Fur, G., Ferrara, P. & Caput, D. (1996). Molecular cloning of a levocabastine-sensitive neurotensin binding site. *FEBS Lett* **386**, 91-4.

Checler, F., Mazella, J., Kitabgi, P. & Vincent, J. P. (1986). High-affinity receptor sites and rapid proteolytic inactivation of neurotensin in primary cultured neurons. *J Neurochem* **47**, 1742-8.

Chuang, D. M. & Costa, E. (1979). beta-Adrenergic receptors of frog erythrocytes. Biochemical sequelae following stimulation with isoproterenol. *Neurochem Res* **4**, 777-93.

Chun, M., Liyanage, U. K., Lisanti, M. P. & Lodish, H. F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A* **91**, 11728-32.

Cook, T., Mesa, K. & Urrutia, R. (1996). Three dynamin-encoding genes are differentially expressed in developing rat brain. *J Neurochem* **67**, 927-31.

Cook, T. A., Urrutia, R. & McNiven, M. A. (1994). Identification of dynamin 2, an isoform ubiquitously expressed in rat tissues. *Proc Natl Acad Sci U S A* **91**, 644-8.

Cowlen, M. S. & Toews, M. L. (1988). Evidence for alpha 1-adrenergic receptor internalization in DDT1 MF-2 cells following exposure to agonists plus protein kinase C activators. *Mol Pharmacol* **34**, 340-6.

Craft, C. M., Whitmore, D. H. & Wiechmann, A. F. (1994). Cone arrestin identified by targeting expression of a functional family. *J Biol Chem* **269**, 4613-9.

Damke, H., Baba, T., Warnock, D. E. & Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol* **127**, 915-34.

David, C., McPherson, P. S., Mundigl, O. & de Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc Natl Acad Sci U S A* **93**, 331-5.

Davis, T. P., Burgess, H. S., Crowell, S., Moody, T. W., Culling-Berglund, A. & Liu, R. H. (1989). Beta-endorphin and neurotensin stimulate in vitro clonal growth of human SCLC cells. *Eur J Pharmacol* **161**, 283-5.

De Camilli, P., Thomas, A., Cofiell, R., Folli, F., Lichte, B., Piccolo, G., Meinck, H. M., Austoni, M., Fassetta, G., Bottazzo, G. & et al. (1993). The synaptic vesicle-associated protein amphiphysin is the 128-kD autoantigen of Stiff-Man syndrome with breast cancer. *J Exp Med* **178**, 2219-23.

de Weerd, W. F. & Leeb-Lundberg, L. M. (1997). Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Galpha subunits Galphaq and Galphai in caveolae in DDT1 MF-2 smooth muscle cells. *J Biol Chem* **272**, 17858-66.

DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D. & Bunnett, N. W. (2000). beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148**, 1267-81.

DeGraff, J. L., Gagnon, A. W., Benovic, J. L. & Orsini, M. J. (1999). Role of arrestins in endocytosis and signaling of alpha2-adrenergic receptor subtypes. *J Biol Chem* **274**, 11253-9.

Dessy, C., Kelly, R. A., Balligand, J. L. & Feron, O. (2000). Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling. *Embo J* **19**, 4272-80.

Di Paolo, G., Sankaranarayanan, S., Wenk, M. R., Daniell, L., Perucco, E., Caldarone, B. J., Flavell, R., Picciotto, M. R., Ryan, T. A., Cremona, O. & De Camilli, P. (2002). Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. *Neuron* **33**, 789-804.

Diatloff-Zito, C., Gordon, A. J., Duchaud, E. & Merlin, G. (1995). Isolation of an ubiquitously expressed cDNA encoding human dynamin II, a member of the large GTP-binding protein family. *Gene* **163**, 301-6.

Dolph, P. J., Ranganathan, R., Colley, N. J., Hardy, R. W., Socolich, M. & Zuker, C. S. (1993). Arrestin function in inactivation of G protein-coupled receptor rhodopsin in vivo. *Science* **260**, 1910-6.

Drmota, T., Gould, G. W. & Milligan, G. (1998). Real time visualization of agonist-mediated redistribution and internalization of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor. *J Biol Chem* **273**, 24000-8.

Dubuc, I., Costentin, J., Terranova, J. P., Barnouin, M. C., Soubrie, P., Le Fur, G., Rostene, W. & Kitabgi, P. (1994). The nonpeptide neurotensin antagonist, SR 48692, used as a tool to reveal putative neurotensin receptor subtypes. *Br J Pharmacol* **112**, 352-4.

Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V. & Simons, K. (1993). Caveolae and sorting in the trans-Golgi network of epithelial cells. *Embo J* **12**, 1597-605.

Elrod-Erickson, M. J. & Kaiser, C. A. (1996). Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. *Mol Biol Cell* **7**, 1043-58.

Evers, B. M., Izukura, M., Chung, D. H., Parekh, D., Yoshinaga, K., Greeley, G. H., Jr., Uchida, T., Townsend, C. M., Jr. & Thompson, J. C. (1992). Neurotensin stimulates growth of colonic mucosa in young and aged rats. *Gastroenterology* **103**, 86-91.

Faure, M. P., Alonso, A., Nouel, D., Gaudriault, G., Dennis, M., Vincent, J. P. & Beaudet, A. (1995). Somatodendritic internalization and perinuclear targeting of neurotensin in the mammalian brain. *J Neurosci* **15**, 4140-7.

Faure, M. P., Labbe-Jullie, C., Cashman, N., Kitabgi, P. & Beaudet, A. (1995). Binding and internalization of neurotensin in hybrid cells derived from septal cholinergic neurons. *Synapse* **20**, 106-16.

Faure, M. P., Shaw, I., Gaudreau, P., Cashman, N. & Beaudet, A. (1992). Binding and internalization of neurotensin in hybrid cells derived from septal cholinergic neurons. *Ann N Y Acad Sci* **668**, 345-7.

Fernandez-Chacon, R., Achiriloaie, M., Janz, R., Albanesi, J. P. & Sudhof, T. C. (2000). SCAMP1 function in endocytosis. *J Biol Chem* **275**, 12752-6.

Feron, O., Smith, T. W., Michel, T. & Kelly, R. A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J Biol Chem* **272**, 17744-8.

Ferrari, S. L., Behar, V., Chorev, M., Rosenblatt, M. & Bisello, A. (1999). Endocytosis of ligand-human parathyroid hormone receptor 1 complexes is protein kinase C-dependent and involves beta-arrestin2. Real-time monitoring by fluorescence microscopy. *J Biol Chem* **274**, 29968-75.

Feurle, G. E., Muller, B. & Rix, E. (1987). Neurotensin induces hyperplasia of the pancreas and growth of the gastric antrum in rats. *Gut* **28**, 19-23.

Fish, K. N., Schmid, S. L. & Damke, H. (2000). Evidence that dynamin-2 functions as a signal-transducing GTPase. *J Cell Biol* **150**, 145-54.

Folli, F., Solimena, M., Cofiell, R., Austoni, M., Tallini, G., Fassetta, G., Bates, D., Cartlidge, N., Bottazzo, G. F., Piccolo, G. & et al. (1993). Autoantibodies to a 128-kd synaptic protein in three women with the stiff-man syndrome and breast cancer. *N Engl J Med* **328**, 546-51.

Fuchs, S., Nakazawa, M., Maw, M., Tamai, M., Oguchi, Y. & Gal, A. (1995). A homozygous 1-base pair deletion in the arrestin gene is a frequent cause of Oguchi disease in Japanese. *Nat Genet* **10**, 360-2.

Gaborik, Z., Szaszak, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J. & Hunyady, L. (2001). Beta-arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. *Mol Pharmacol* **59**, 239-47.

Gagnon, A. W., Kallal, L. & Benovic, J. L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down- regulation of the beta2-adrenergic receptor. *J Biol Chem* **273**, 6976-81.

Gardner, B., Liu, Z. F., Jiang, D. & Sibley, D. R. (2001). The role of phosphorylation/dephosphorylation in agonist-induced desensitization of D1 dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation. *Mol Pharmacol* **59**, 310-21.

Garver, D. L., Bissette, G., Yao, J. K. & Nemeroff, C. B. (1991). Relation of CSF neurotensin concentrations to symptoms and drug response of psychotic patients. *Am J Psychiatry* **148**, 484-8.

Gaudriault, G. & Vincent, J. P. (1992). Selective labeling of alpha- or epsilon-amino groups in peptides by the Bolton-Hunter reagent. *Peptides* **13**, 1187-92.

Goedert, M., Pinnock, R. D., Downes, C. P., Mantyh, P. W. & Emson, P. C. (1984). Neurotensin stimulates inositol phospholipid hydrolysis in rat brain slices. *Brain Res* **323**, 193-7.

Gold, E. S., Morrissette, N. S., Underhill, D. M., Guo, J., Bassetti, M. & Aderem, A. (2000). Amphiphysin IIm, a novel amphiphysin II isoform, is required for macrophage phagocytosis. *Immunity* **12**, 285-92.

Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A. & Aderem, A. (1999). Dynamin 2 is required for phagocytosis in macrophages. *J Exp Med* **190**, 1849-56.

Goldman, P. S., Schlador, M. L., Shapiro, R. A. & Nathanson, N. M. (1996). Identification of a region required for subtype-specific agonist- induced sequestration of the m2 muscarinic acetylcholine receptor. *J Biol Chem* **271**, 4215-22.

Goldman, R., Bar-Shavit, Z., Shezen, E., Terry, S. & Blumberg, S. (1982). Enhancement of phagocytosis by neurotensin, a newly found biological activity of the neuropeptide. *Adv Exp Med Biol* **155**, 133-41.

Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L. & Keen, J. H. (1997). Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J Biol Chem* **272**, 15017-22.

Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. & Benovic, J. L. (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2- adrenergic receptor. *Nature* **383**, 447-50.

Grabs, D., Slepnev, V. I., Songyang, Z., David, C., Lynch, M., Cantley, L. C. & De Camilli, P. (1997). The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. *J Biol Chem* **272**, 13419-25.

Grady, E. F., Slice, L. W., Brant, W. O., Walsh, J. H., Payan, D. G. & Bunnett, N. W. (1995). Direct observation of endocytosis of gastrin releasing peptide and its receptor. *J Biol Chem* **270**, 4603-11.

Greene, L. E. & Eisenberg, E. (1990). Dissociation of clathrin from coated vesicles by the uncoating ATPase. *J Biol Chem* **265**, 6682-7.

Grigliatti, T. A., Hall, L., Rosenbluth, R. & Suzuki, D. T. (1973). Temperature-sensitive mutations in Drosophila melanogaster. XIV. A selection of immobile adults. *Mol Gen Genet* **120**, 107-14.

Guipponi, M., Scott, H. S., Chen, H., Schebesta, A., Rossier, C. & Antonarakis, S. E. (1998). Two isoforms of a human intersectin (ITSN) protein are produced by brain-specific alternative splicing in a stop codon. *Genomics* **53**, 369-76.

Gully, D., Canton, M., Boigegrain, R., Jeanjean, F., Molimard, J. C., Poncelet, M., Gueudet, C., Heaulme, M., Leyris, R., Brouard, A. & et al. (1993). Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proc Natl Acad Sci U S A* **90**, 65-9.

Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M. & Benovic, J. L. (1995). Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, beta 2- adrenergic, and m2 muscarinic cholinergic receptors. *J Biol Chem* **270**, 720-31.

Hazum, E., Cuatrecasas, P., Marian, J. & Conn, P. M. (1980). Receptor-mediated internalization of fluorescent gonadotropin-releasing hormone by pituitary gonadotropes. *Proc Natl Acad Sci U S A* **77**, 6692-5.

Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L. & Eidne, K. A. (1998). Gonadotropin-releasing hormone receptors with intracellular carboxyl- terminal tails undergo acute desensitization of total inositol phosphate production and exhibit accelerated internalization kinetics. *J Biol Chem* **273**, 11472-7.

Henley, J. R., Krueger, E. W., Oswald, B. J. & McNiven, M. A. (1998). Dynamin-mediated internalization of caveolae. *J Cell Biol* **141**, 85-99.

Hermans, E., Octave, J. N. & Maloteaux, J. M. (1994). Receptor mediated internalization of neurotensin in transfected Chinese hamster ovary cells. *Biochem Pharmacol* **47**, 89-91.

Herskovits, J. S., Burgess, C. C., Obar, R. A. & Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. *J Cell Biol* **122**, 565-78.

Hinshaw, J. E. & Schmid, S. L. (1995). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* **374**, 190-2.

Hussain, N. K., Yamabhai, M., Ramjaun, A. R., Guy, A. M., Baranes, D., O'Bryan, J. P., Der, C. J., Kay, B. K. & McPherson, P. S. (1999). Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J Biol Chem* **274**, 15671-7.

Imaizumi, T., Osugi, T., Misaki, N., Uchida, S. & Yoshida, H. (1989). Heterologous desensitization of bradykinin-induced phosphatidylinositol response and Ca2+ mobilization by neurotensin in NG108-15 cells. *Eur J Pharmacol* **161**, 203-8.

Innamorati, G., Sadeghi, H. M., Tran, N. T. & Birnbaumer, M. (1998). A serine cluster prevents recycling of the V2 vasopressin receptor. *Proc Natl Acad Sci U S A* **95**, 2222-6.

Iwase, K., Evers, B. M., Hellmich, M. R., Kim, H. J., Higashide, S., Gully, D., Thompson, J. C. & Townsend, C. M., Jr. (1997). Inhibition of neurotensin-induced pancreatic carcinoma growth by a nonpeptide neurotensin receptor antagonist, SR48692. *Cancer* **79**, 1787-93.

Iwase, K., Evers, B. M., Hellmich, M. R., Kim, H. J., Higashide, S., Gully, D. & Townsend, C. M., Jr. (1996). Indirect inhibitory effect of a neurotensin receptor antagonist on human colon cancer (LoVo) growth. *Surg Oncol* **5**, 245-51.

Jackson, A. P., Seow, H. F., Holmes, N., Drickamer, K. & Parham, P. (1987). Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments. *Nature* **326**, 154-9.

Jones, S. M., Howell, K. E., Henley, J. R., Cao, H. & McNiven, M. A. (1998). Role of dynamin in the formation of transport vesicles from the trans- Golgi network. *Science* **279**, 573-7.

Kadlec, L. & Pendergast, A. M. (1997). The amphiphysin-like protein 1 (ALP1) interacts functionally with the cABL tyrosine kinase and may play a role in cytoskeletal regulation. *Proc Natl Acad Sci U S A* **94**, 12390-5.

Kalivas, P. W., Burgess, S. K., Nemeroff, C. B. & Prange, A. J., Jr. (1983). Behavioral and neurochemical effects of neurotensin microinjection into the ventral tegmental area of the rat. *Neuroscience* **8**, 495-505.

Kalivas, P. W., Nemeroff, C. B., Miller, J. S. & Prange, A. J., Jr. (1985). Microinjection of neurotensin into the ventral tegmental area produces hypothermia: evaluation of dopaminergic mediation. *Brain Res* **326**, 219-27.

Kamen, B. A., Wang, M. T., Streckfuss, A. J., Peryea, X. & Anderson, R. G. (1988). Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J Biol Chem* **263**, 13602-9.

Kapuscinski, M., Shulkes, A., Read, D. & Hardy, K. J. (1990). Expression of neurotensin in endocrine tumors. *J Clin Endocrinol Metab* **70**, 100-6.

Keen, J. H. (1990). Clathrin and associated assembly and disassembly proteins. *Annu Rev Biochem* **59**, 415-38.

Kessell, I., Holst, B. D. & Roth, T. F. (1989). Membranous intermediates in endocytosis are labile, as shown in a temperature-sensitive mutant. *Proc Natl Acad Sci U S A* **86**, 4968-72.

Kinkead, B., Binder, E. B. & Nemeroff, C. B. (1999). Does neurotensin mediate the effects of antipsychotic drugs? *Biol Psychiatry* **46**, 340-51.

Kirchhausen, T., Harrison, S. C. & Heuser, J. (1986). Configuration of clathrin trimers: evidence from electron microscopy. *J Ultrastruct Mol Struct Res* **94**, 199-208.

Kirchhausen, T. & Toyoda, T. (1993). Immunoelectron microscopic evidence for the extended conformation of light chains in clathrin trimers. *J Biol Chem* **268**, 10268-73.

Kitabgi, P., Rostene, W., Dussaillant, M., Schotte, A., Laduron, P. M. & Vincent, J. P. (1987). Two populations of neurotensin binding sites in murine brain: discrimination by the antihistamine levocabastine reveals markedly different radioautographic distribution. *Eur J Pharmacol* **140**, 285-93.

Kittler, J. T., Delmas, P., Jovanovic, J. N., Brown, D. A., Smart, T. G. & Moss, S. J. (2000). Constitutive endocytosis of GABAA receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J Neurosci* **20**, 7972-7.

Koenig, J. A., Kaur, R., Dodgeon, I., Edwardson, J. M. & Humphrey, P. P. (1998). Fates of endocytosed somatostatin sst2 receptors and associated agonists. *Biochem J* **336**, 291-8.

Kozlov, M. M. (1999). Dynamin: possible mechanism of "Pinchase" action. *Biophys J* **77**, 604-16.

Krudewig, R., Langer, B., Vogler, O., Markschies, N., Erl, M., Jakobs, K. H. & van Koppen, C. J. (2000). Distinct internalization of M2 muscarinic acetylcholine receptors confers selective and long-lasting desensitization of signaling to phospholipase C. *J Neurochem* **74**, 1721-30.

Kuhn, H. & Wilden, U. (1987). Deactivation of photoactivated rhodopsin by rhodopsin-kinase and arrestin. *J Recept Res* **7**, 283-98.

Lahti, R. A., Cochrane, E. V., Roberts, R. C., Conley, R. R. & Tamminga, C. A. (1998). [3H]Neurotensin receptor densities in human postmortem brain tissue obtained from normal and schizophrenic persons. An autoradiographic study. *J Neural Transm* **105**, 507-16.

Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S. & Caron, M. G. (2000). The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. *J Biol Chem* **275**, 23120-6.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G. & Barak, L. S. (1999). The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci U S A* **96**, 3712-7.

Lazari, M. F., Bertrand, J. E., Nakamura, K., Liu, X., Krupnick, J. G., Benovic, J. L. & Ascoli, M. (1998). Mutation of individual serine residues in the C-terminal tail of the lutropin/choriogonadotropin receptor reveal distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization. *J Biol Chem* **273**, 18316-24.

Le Gouill, C., Parent, J. L., Rola-Pleszczynski, M. & Stankova, J. (1997). Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor. *J Biol Chem* **272**, 21289-95.

Lee, A., Frank, D. W., Marks, M. S. & Lemmon, M. A. (1999). Dominant-negative inhibition of receptor-mediated endocytosis by a dynamin-1 mutant with a defective pleckstrin homology domain. *Curr Biol* **9**, 261-4.

Lee, K. B., Pals-Rylaarsdam, R., Benovic, J. L. & Hosey, M. M. (1998). Arrestin-independent internalization of the m1, m3, and m4 subtypes of muscarinic cholinergic receptors. *J Biol Chem* **273**, 12967-72.

Lemaire, I. (1988). Neurotensin enhances IL-1 production by activated alveolar macrophages. *J Immunol* **140**, 2983-8.

Lenkei, Z., Beaudet, A., Chartrel, N., De Mota, N., Irinopoulou, T., Braun, B., Vaudry, H. & Llorens-Cortes, C. (2000). A highly sensitive quantitative cytosensor technique for the identification of receptor ligands in tissue extracts. *J Histochem Cytochem* **48**, 1553-64.

Leprince, C., Romero, F., Cussac, D., Vayssiere, B., Berger, R., Tavitian, A. & Camonis, J. H. (1997). A new member of the amphiphysin family connecting endocytosis and signal transduction pathways. *J Biol Chem* **272**, 15101-5.

Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H. & Cosson, P. (1994). Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199-207.

Leventis, P. A., Chow, B. M., Stewart, B. A., Iyengar, B., Campos, A. R. & Boulianne, G. L. (2001). Drosophila Amphiphysin is a post-synaptic protein required for normal locomotion but not endocytosis. *Traffic* **2**, 839-50.

Li, J. G., Luo, L. Y., Krupnick, J. G., Benovic, J. L. & Liu-Chen, L. Y. (1999). U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J Biol Chem* **274**, 12087-94.

Lichte, B., Veh, R. W., Meyer, H. E. & Kilimann, M. W. (1992). Amphiphysin, a novel protein associated with synaptic vesicles. *Embo J* **11**, 2521-30.

Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A. & Lefkowitz, R. J. (1997). Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1. *J Biol Chem* **272**, 31051-7.

Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J. P., Caron, M. G. & Lefkowitz, R. J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J Biol Chem* **267**, 8558-64.

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G. & Lefkowitz, R. J. (1990). beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**, 1547-50.

Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G. & Lefkowitz, R. J. (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**, 655-61.

Mahaffey, D. T., Peeler, J. S., Brodsky, F. M. & Anderson, R. G. (1990). Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. *J Biol Chem* **265**, 16514-20.

Mai, J. K., Triepel, J. & Metz, J. (1987). Neurotensin in the human brain. *Neuroscience* **22**, 499-524.

Mann, P. T., Southwell, B. R. & Furness, J. B. (1999). Internalization of the neurokinin 1 receptor in rat myenteric neurons. *Neuroscience* **91**, 353-62.

Maoret, J. J., Anini, Y., Rouyer-Fessard, C., Gully, D. & Laburthe, M. (1999). Neurotensin and a non-peptide neurotensin receptor antagonist control human colon cancer cell growth in cell culture and in cells xenografted into nude mice. *Int J Cancer* **80**, 448-54.

Marks, O. H., Kirchhausen T, Bonifacino JS (1997). Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores. *Trends in Cell Biology* **7**, 124 - 128.

Marsh, M. & Helenius, A. (1980). Adsorptive endocytosis of Semliki Forest virus. *J Mol Biol* **142**, 439-54.

Martina, J. A., Bonangelino, C. J., Aguilar, R. C. & Bonifacino, J. S. (2001). Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J Cell Biol* **153**, 1111-20.

Maxfield, F. R., Willingham, M. C., Davies, P. J. & Pastan, I. (1979). Amines inhibit the clustering of alpha2-macroglobulin and EGF on the fibroblast cell surface. *Nature* **277**, 661-3.

Mazella, J., Botto, J. M., Guillemare, E., Coppola, T., Sarret, P. & Vincent, J. P. (1996). Structure, functional expression, and cerebral localization of the levocabastine-sensitive neurotensin/neuromedin N receptor from mouse brain. *J Neurosci* **16**, 5613-20.

Mazella, J., Leonard, K., Chabry, J., Kitabgi, P., Vincent, J. P. & Beaudet, A. (1991). Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. *Brain Res* **564**, 249-55.

Mazella, J., Zsurger, N., Navarro, V., Chabry, J., Kaghad, M., Caput, D., Ferrara, P., Vita, N., Gully, D., Maffrand, J. P. & Vincent, J. P. (1998). The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein- coupled receptor. *J Biol Chem* **273**, 26273-6.

McConalogue, K., Dery, O., Lovett, M., Wong, H., Walsh, J. H., Grady, E. F. & Bunnett, N. W. (1999). Substance P-induced trafficking of beta-arrestins. The role of beta- arrestins in endocytosis of the neurokinin-1 receptor. *J Biol Chem* **274**, 16257-68.

McMahon, H. T., Wigge, P. & Smith, C. (1997). Clathrin interacts specifically with amphiphysin and is displaced by dynamin. *FEBS Lett* **413**, 319-22.

McNiven, M. A., Kim, L., Krueger, E. W., Orth, J. D., Cao, H. & Wong, T. W. (2000). Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape. *J Cell Biol* **151**, 187-98.

Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G. & Barak, L. S. (1997). Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta- arrestin determine kinetics of internalization. *Mol Pharmacol* **51**, 800-8.

Miller, W. E. & Lefkowitz, R. J. (2001). Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr Opin Cell Biol* **13**, 139-45.

Miller, W. E., McDonald, P. H., Cai, S. F., Field, M. E., Davis, R. J. & Lefkowitz, R. J. (2001). Identification of a motif in the carboxyl terminus of beta -arrestin2 responsible for activation of JNK3. *J Biol Chem* **276**, 27770-7.

Monier, S., Dietzen, D. J., Hastings, W. R., Lublin, D. M. & Kurzchalia, T. V. (1996). Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. *FEBS Lett* **388**, 143-9.

Moody, T. W., Carney, D. N., Cuttitta, F., Quattrocchi, K. & Minna, J. D. (1985). High affinity receptors for bombesin/GRP-like peptides on human small cell lung cancer. *Life Sci* **37**, 105-13.

Moody, T. W., Chiles, J., Casibang, M., Moody, E., Chan, D. & Davis, T. P. (2001). SR48692 is a neurotensin receptor antagonist which inhibits the growth of small cell lung cancer cells. *Peptides* **22**, 109-15.

Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J. A., Carsrud, N. D., Dickey, B. F. & Knoll, B. J. (1995). Ligand-stimulated beta 2-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. *J Cell Sci* **108**, 2983-91.

Muhlberg, A. B., Warnock, D. E. & Schmid, S. L. (1997). Domain structure and intramolecular regulation of dynamin GTPase. *Embo J* **16**, 6676-83.

Mundell, S. J. & Kelly, E. (1998). The effect of inhibitors of receptor internalization on the desensitization and resensitization of three Gs-coupled receptor responses. *Br J Pharmacol* **125**, 1594-600.

Murray, S. R., Evans, C. J. & von Zastrow, M. (1998). Phosphorylation is not required for dynamin-dependent endocytosis of a truncated mutant opioid receptor. *J Biol Chem* **273**, 24987-91.

Narita, K., Tsuruhara, T., Koenig, J. H. & Ikeda, K. (1989). Membrane pinch-off and reinsertion observed in living cells of Drosophila. *J Cell Physiol* **141**, 383-91.

Navarro, A., Zapata, R., Canela, E. I., Mallol, J., Lluis, C. & Franco, R. (1999). Epidermal growth factor (EGF)-induced up-regulation and agonist- and antagonist-induced desensitization and internalization of A1 adenosine receptors in a pituitary-derived cell line. *Brain Res* **816**, 47-57.

Neer, E. J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80**, 249-57.

Nemeroff, C. B. (1980). Neurotensin: perchance an endogenous neuroleptic? *Biol Psychiatry* **15**, 283-302.

Nemeroff, C. B. (1986). The interaction of neurotensin with dopaminergic pathways in the central nervous system: basic neurobiology and implications for the pathogenesis and treatment of schizophrenia. *Psychoneuroendocrinology* **11**, 15-37.

Newman-Smith, E. D., Shurland, D. L. & van der Bliek, A. M. (1997). Assignment of the dynamin-1 gene (DNM1) to human chromosome 9q34 by fluorescence in situ hybridization and somatic cell hybrid analysis. *Genomics* **41**, 286-9.

Ng, G. Y., Trogadis, J., Stevens, J., Bouvier, M., O'Dowd, B. F. & George, S. R. (1995). Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D1 receptor internalization. *Proc Natl Acad Sci U S A* **92**, 10157-61.

Nouel, D., Faure, M. P., St Pierre, J. A., Alonso, R., Quirion, R. & Beaudet, A. (1997). Differential binding profile and internalization process of neurotensin via neuronal and glial receptors. *J Neurosci* **17**, 1795-803.

Nouel, D., Sarret, P., Vincent, J. P., Mazella, J. & Beaudet, A. (1999). Pharmacological, molecular and functional characterization of glial neurotensin receptors. *Neuroscience* **94**, 1189-97.

Nussenzveig, D. R., Heinflink, M. & Gershengorn, M. C. (1993). Agonist-stimulated internalization of the thyrotropin-releasing hormone receptor is dependent on two domains in the receptor carboxyl terminus. *J Biol Chem* **268**, 2389-92.

Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S. & Caron, M. G. (1999). Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* **274**, 32248-57.

Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G. & Barak, L. S. (2000). Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* **275**, 17201-10.

Oh, P., McIntosh, D. P. & Schnitzer, J. E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J Cell Biol* **141**, 101-14.

Ohno, H., Stewart, J., Fournier, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T. & Bonifacino, J. S. (1995). Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269**, 1872-5.

Okamoto, P. M., Tripet, B., Litowski, J., Hodges, R. S. & Vallee, R. B. (1999). Multiple distinct coiled-coils are involved in dynamin self-assembly. *J Biol Chem* **274**, 10277-86.

Orsini, M. J., Parent, J. L., Mundell, S. J., Benovic, J. L. & Marchese, A. (1999). Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. *J Biol Chem* **274**, 31076-86.

Owen, D. J., Wigge, P., Vallis, Y., Moore, J. D., Evans, P. R. & McMahon, H. T. (1998). Crystal structure of the amphiphysin-2 SH3 domain and its role in the prevention of dynamin ring formation. *Embo J* **17**, 5273-85.

Palacios, J. M. & Kuhar, M. J. (1981). Neurotensin receptors are located on dopamine-containing neurones in rat midbrain. *Nature* **294**, 587-9.

Palade, G. E. (1953). The fine structure of blood capillaries. *Journal of Applied Physics* **24**, 1424.

Park, J. S., Park, W. Y., Cho, K. A., Kim, D. I., Jhun, B. H., Kim, S. R. & Park, S. C. (2001). Down-regulation of amphiphysin-1 is responsible for reduced receptor-mediated endocytosis in the senescent cells. *Faseb J* **15**, 1625-7.

Payne, G. S. (1990). Genetic analysis of clathrin function in yeast. *J Membr Biol* **116**, 93-105.

Pearse, B. M. (1975). Coated vesicles from pig brain: purification and biochemical characterization. *J Mol Biol* **97**, 93-8.

Pearse, B. M. & Robinson, M. S. (1990). Clathrin, adaptors, and sorting. *Annu Rev Cell Biol* **6**, 151-71.

Peters, K. R., Carley, W. W. & Palade, G. E. (1985). Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. *J Cell Biol* **101**, 2233-8.

Petersen, C. M., Nielsen, M. S., Nykjaer, A., Jacobsen, L., Tommerup, N., Rasmussen, H. H., Roigaard, H., Gliemann, J., Madsen, P. & Moestrup, S. K. (1997). Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J Biol Chem* **272**, 3599-605.

Pfister, C., Chabre, M., Plouet, J., Tuyen, V. V., De Kozak, Y., Faure, J. P. & Kuhn, H. (1985). Retinal S antigen identified as the 48K protein regulating light-dependent phosphodiesterase in rods. *Science* **228**, 891-3.

Pippig, S., Andexinger, S. & Lohse, M. J. (1995). Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**, 666-76.

Pizard, A., Blaukat, A., Muller-Esterl, W., Alhenc-Gelas, F. & Rajerison, R. M. (1999). Bradykinin-induced internalization of the human B2 receptor requires phosphorylation of three serine and two threonine residues at its carboxyl tail. *J Biol Chem* **274**, 12738-47.

Poinot-Chazel, C., Portier, M., Bouaboula, M., Vita, N., Pecceu, F., Gully, D., Monroe, J. G., Maffrand, J. P., Le Fur, G. & Casellas, P. (1996). Activation of mitogen-activated protein kinase couples neurotensin receptor stimulation to induction of the primary response gene Krox-24. *Biochem J* **320**, 145-51.

Poodry, C. A., Hall, L. & Suzuki, D. T. (1973). Developmental properties of Shibire: a pleiotropic mutation affecting larval and adult locomotion and development. *Dev Biol* **32**, 373-86.

Powell, K. A. & Robinson, P. J. (1995). Dephosphin/dynamin is a neuronal phosphoprotein concentrated in nerve terminals: evidence from rat cerebellum. *Neuroscience* **64**, 821-33.

Prado, G. N., Suzuki, H., Wilkinson, N., Cousins, B. & Navarro, J. (1996). Role of the C terminus of the interleukin 8 receptor in signal transduction and internalization. *J Biol Chem* **271**, 19186-90.

Pucharcos, C., Estivill, X. & de la Luna, S. (2000). Intersectin 2, a new multimodular protein involved in clathrin-mediated endocytosis. *FEBS Lett* **478**, 43-51.

Quirion, R. (1983). Interactions between neurotensin and dopamine in the brain: an overview. *Peptides* **4**, 609-15.

Quirion, R., Chiueh, C. C., Everist, H. D. & Pert, A. (1985). Comparative localization of neurotensin receptors on nigrostriatal and mesolimbic dopaminergic terminals. *Brain Res* **327**, 385-9.

Ramjaun, A. R., Micheva, K. D., Bouchelet, I. & McPherson, P. S. (1997). Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J Biol Chem* **272**, 16700-6.

Rankin, M. L., Alvania, R. S., Gleason, E. L. & Bruch, R. C. (1999). Internalization of G protein-coupled receptors in single olfactory receptor neurons. *J Neurochem* **72**, 541-8.

Ray, P., Middleton, W. & Berman, J. D. (1989). Mechanism of agonist-induced down-regulation and subsequent recovery of muscarinic acetylcholine receptors in a clonal neuroblastoma x glioma hybrid cell line. *J Neurochem* **52**, 402-9.

Razzaq, A., Robinson, I. M., McMahon, H. T., Skepper, J. N., Su, Y., Zelhof, A. C., Jackson, A. P., Gay, N. J. & O'Kane, C. J. (2001). Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in Drosophila. *Genes Dev* **15**, 2967-79.

Roettger, B. F., Ghanekar, D., Rao, R., Toledo, C., Yingling, J., Pinon, D. & Miller, L. J. (1997). Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* **51**, 357-62.

Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M. & Miller, L. J. (1995). Dual pathways of internalization of the cholecystokinin receptor. *J Cell Biol* **128**, 1029-41.

Rostene, W. H. & Alexander, M. J. (1997). Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* **18**, 115-73.

Roth, T. F., Porter, K.R. (1964). Yolk protein uptake in the oocyte of the mosquito Aedes aegypti. *Journal of Cell Biology* **20**, 313-332.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. & Anderson, R. G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-82.

Rowe, T., Aridor, M., McCaffery, J. M., Plutner, H., Nuoffer, C. & Balch, W. E. (1996). COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI. *J Cell Biol* **135**, 895-911.

Sakamuro, D., Elliott, K. J., Wechsler-Reya, R. & Prendergast, G. C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. *Nat Genet* **14**, 69-77.

Sarret, P. (2001). ROLE OF AMPHIPHYSIN II IN SOMATOSTATIN RECEPTOR TRAFFICKING IN NEUROENDOCRINE CELLS.

Scaife, R. & Margolis, R. L. (1990). Biochemical and immunochemical analysis of rat brain dynamin interaction with microtubules and organelles in vivo and in vitro. *J Cell Biol* **111**, 3023-33.

Scherer, N. M. & Nathanson, N. M. (1990). Differential regulation by agonist and phorbol ester of cloned m1 and m2 muscarinic acetylcholine receptors in mouse Y1 adrenal cells and in Y1 cells deficient in cAMP-dependent protein kinase. *Biochemistry* **29**, 8475-83.

Schlador, M. L., Grubbs, R. D. & Nathanson, N. M. (2000). Multiple topological domains mediate subtype-specific internalization of the M2 muscarinic acetylcholine receptor. *J Biol Chem* **275**, 23295-302.

Schlossman, D. M., Schmid, S. L., Braell, W. A. & Rothman, J. E. (1984). An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J Cell Biol* **99**, 723-33.

Schmid, S. L. & Damke, H. (1995). Coated vesicles: a diversity of form and function. *Faseb J* **9**, 1445-53.

Schmidlin, F., Dery, O., DeFea, K. O., Slice, L., Patierno, S., Sternini, C., Grady, E. F. & Bunnett, N. W. (2001). Dynamin and Rab5a-dependent trafficking and signaling of the neurokinin 1 receptor. *J Biol Chem* **276**, 25427-37.

Schnitzer, J. E., Oh, P. & McIntosh, D. P. (1996). Role of GTP hydrolysis in fission of caveolae directly from plasma membranes. *Science* **274**, 239-42.

Schnitzer, J. E., Oh, P., Pinney, E. & Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* **127**, 1217-32.

Seethalakshmi, L., Mitra, S. P., Dobner, P. R., Menon, M. & Carraway, R. E. (1997). Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. *Prostate* **31**, 183-92.

Sehgal, I., Powers, S., Huntley, B., Powis, G., Pittelkow, M. & Maihle, N. J. (1994). Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. *Proc Natl Acad Sci U S A* **91**, 4673-7.

Sengar, A. S., Wang, W., Bishay, J., Cohen, S. & Egan, S. E. (1999). The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *Embo J* **18**, 1159-71.

Severs, N. J. (1988). Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact? *J Cell Sci* **90**, 341-8.

Sharma, R. P., Janicak, P. G., Bissette, G. & Nemeroff, C. B. (1997). CSF neurotensin concentrations and antipsychotic treatment in schizophrenia and schizoaffective disorder. *Am J Psychiatry* **154**, 1019-21.

Shi, W. X. & Bunney, B. S. (1992). Actions of neurotensin: a review of the electrophysiological studies. *Ann N Y Acad Sci* **668**, 129-45.

Shpetner, H. S. & Vallee, R. B. (1989). Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell* **59**, 421-32.

Shpetner, H. S. & Vallee, R. B. (1992). Dynamin is a GTPase stimulated to high levels of activity by microtubules. *Nature* **355**, 733-5.

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P. & Brodin, L. (1997). Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. *Science* **276**, 259-63.

Signoret, N., Oldridge, J., Pelchen-Matthews, A., Klasse, P. J., Tran, T., Brass, L. F., Rosenkilde, M. M., Schwartz, T. W., Holmes, W., Dallas, W., Luther, M. A., Wells, T. N., Hoxie, J. A. & Marsh, M. (1997). Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J Cell Biol* **139**, 651-64.

Singleton, D. R., Wu, T. T. & Castle, J. D. (1997). Three mammalian SCAMPs (secretory carrier membrane proteins) are highly related products of distinct genes having similar subcellular distributions. *J Cell Sci* **110**, 2099-107.

Slepnev, V. I., Ochoa, G. C., Butler, M. H., Grabs, D. & Camilli, P. D. (1998). Role of phosphorylation in regulation of the assembly of endocytic coat complexes. *Science* **281**, 821-4.

Smirnova, E., Shurland, D. L., Newman-Smith, E. D., Pishvaee, B. & van der Bliek, A. M. (1999). A model for dynamin self-assembly based on binding between three different protein domains. *J Biol Chem* **274**, 14942-7.

Smith, W. C., Milam, A. H., Dugger, D., Arendt, A., Hargrave, P. A. & Palczewski, K. (1994). A splice variant of arrestin. Molecular cloning and localization in bovine retina. *J Biol Chem* **269**, 15407-10.

Snider, R. M., Forray, C., Pfenning, M. & Richelson, E. (1986). Neurotensin stimulates inositol phospholipid metabolism and calcium mobilization in murine neuroblastoma clone N1E-115. *J Neurochem* **47**, 1214-8.

Sontag, J. M., Fykse, E. M., Ushkaryov, Y., Liu, J. P., Robinson, P. J. & Sudhof, T. C. (1994). Differential expression and regulation of multiple dynamins. *J Biol Chem* **269**, 4547-54.

Souaze, F., Rostene, W. & Forgez, P. (1997). Neurotensin agonist induces differential regulation of neurotensin receptor mRNA. Identification of distinct transcriptional and post- transcriptional mechanisms. *J Biol Chem* **272**, 10087-94.

Southwell, B. R., Seybold, V. S., Woodman, H. L., Jenkinson, K. M. & Furness, J. B. (1998). Quantitation of neurokinin 1 receptor internalization and recycling in guinea-pig myenteric neurons. *Neuroscience* **87**, 925-31.

Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M. & Kay, B. K. (1996). Cloning of ligand targets: systematic isolation of SH3 domain- containing proteins. *Nat Biotechnol* **14**, 741-4.

Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A. & Kay, B. K. (1996). Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCgamma, Crk, and Grb2. *Proc Natl Acad Sci U S A* **93**, 1540-4.

Stowell, M. H., Marks, B., Wigge, P. & McMahon, H. T. (1999). Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring. *Nat Cell Biol* **1**, 27-32.

Superti, F., Seganti, L., Ruggeri, F. M., Tinari, A., Donelli, G. & Orsi, N. (1987). Entry pathway of vesicular stomatitis virus into different host cells. *J Gen Virol* **68**, 387-99.

Surya, A., Stadel, J. M. & Knox, B. E. (1998). Evidence for multiple, biochemically distinguishable states in the G protein-coupled receptor, rhodopsin. *Trends Pharmacol Sci* **19**, 243-7.

Sweitzer, S. M. & Hinshaw, J. E. (1998). Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* **93**, 1021-9.

Takei, K., McPherson, P. S., Schmid, S. L. & De Camilli, P. (1995). Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. *Nature* **374**, 186-90.

Takei, K., Slepnev, V. I. & De Camilli, P. (2001). Interactions of dynamin and amphiphysin with liposomes. *Methods Enzymol* **329**, 478-86.

Takei, K., Slepnev, V. I., Haucke, V. & De Camilli, P. (1999). Functional partnership between amphiphysin and dynamin in clathrin- mediated endocytosis. *Nat Cell Biol* **1**, 33-9.

Tanaka, K., Masu, M. & Nakanishi, S. (1990). Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* **4**, 847-54.

Trejo, J., Altschuler, Y., Fu, H. W., Mostov, K. E. & Coughlin, S. R. (2000). Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *J Biol Chem* **275**, 31255-65.

Trowbridge, I. S., Collawn, J. F. & Hopkins, C. R. (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu Rev Cell Biol* **9**, 129-61.

Tsutsui, K., Maeda, Y., Seki, S. & Tokunaga, A. (1997). cDNA cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. *Biochem Biophys Res Commun* **236**, 178-83.

Ungewickell, E. & Branton, D. (1981). Assembly units of clathrin coats. *Nature* **289**, 420-2.

Vallis, Y., Wigge, P., Marks, B., Evans, P. R. & McMahon, H. T. (1999). Importance of the pleckstrin homology domain of dynamin in clathrin- mediated endocytosis. *Curr Biol* **9**, 257-60.

van Dam, E. M. & Stoorvogel, W. (2002). Dynamin-dependent Transferrin Receptor Recycling by Endosome-derived Clathrin-coated Vesicles. *Mol Biol Cell* **13**, 169-82.

van der Bliek, A. M. & Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature* **351**, 411-4.

Vandenbulcke, F., Nouel, D., Vincent, J. P., Mazella, J. & Beaudet, A. (2000). Ligand-induced internalization of neurotensin in transfected COS-7 cells: differential intracellular trafficking of ligand and receptor. *J Cell Sci* **113**, 2963-75.

Vanisberg, M. A., Maloteaux, J. M., Octave, J. N. & Laduron, P. M. (1991). Rapid agonist-induced decrease of neurotensin receptors from the cell surface in rat cultured neurons. *Biochem Pharmacol* **42**, 2265-74.

Vickery, R. G. & von Zastrow, M. (1999). Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol* **144**, 31-43.

Vigers, G. P., Crowther, R. A. & Pearse, B. M. (1986). Location of the 100 kd-50 kd accessory proteins in clathrin coats. *Embo J* **5**, 2079-85.

Vincent, J. P. (1995). Neurotensin receptors: binding properties, transduction pathways, and structure. *Cell Mol Neurobiol* **15**, 501-12.

Vita, N., Laurent, P., Lefort, S., Chalon, P., Dumont, X., Kaghad, M., Gully, D., Le Fur, G., Ferrara, P. & Caput, D. (1993). Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor. *FEBS Lett* **317**, 139-42.

Vogler, O., Bogatkewitsch, G. S., Wriske, C., Krummenerl, P., Jakobs, K. H. & van Koppen, C. J. (1998). Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin. Distinct sequestration of m2 receptors. *J Biol Chem* **273**, 12155-60.

Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K. H. & van Koppen, C. J. (1999). Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin. *J Biol Chem* **274**, 12333-8.

Volchuk, A., Narine, S., Foster, L. J., Grabs, D., De Camilli, P. & Klip, A. (1998). Perturbation of dynamin II with an amphiphysin SH3 domain increases GLUT4 glucose transporters at the plasma membrane in 3T3-L1 adipocytes. Dynamin II participates in GLUT4 endocytosis. *J Biol Chem* **273**, 8169-76.

Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G. & Shetzline, M. A. (1999). Properties of secretin receptor internalization differ from those of the beta(2)-adrenergic receptor. *J Biol Chem* **274**, 31515-23.

Wang, H. L. (2000). A cluster of Ser/Thr residues at the C-terminus of mu-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization. *Neuropharmacology* **39**, 353-63.

Wang, L. H., Sudhof, T. C. & Anderson, R. G. (1995). The appendage domain of alpha-adaptin is a high affinity binding site for dynamin. *J Biol Chem* **270**, 10079-83.

Warnock, D. E., Baba, T. & Schmid, S. L. (1997). Ubiquitously expressed dynamin-II has a higher intrinsic GTPase activity and a greater propensity for self-assembly than neuronal dynamin-I. *Mol Biol Cell* **8**, 2553-62.

Weiss, S., Schmidt, B. H., Sebben, M., Kemp, D. E., Bockaert, J. & Sladeczek, F. (1988). Neurotransmitter-induced inositol phosphate formation in neurons in primary culture. *J Neurochem* **50**, 1425-33.

Werbonat, Y., Kleutges, N., Jakobs, K. H. & van Koppen, C. J. (2000). Essential role of dynamin in internalization of M2 muscarinic acetylcholine and angiotensin AT1A receptors. *J Biol Chem* **275**, 21969-74.

Wigge, P., Vallis, Y. & McMahon, H. T. (1997). Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr Biol* **7**, 554-60.

Wolf, S. S., Hyde, T. M., Saunders, R. C., Herman, M. M., Weinberger, D. R. & Kleinman, J. E. (1995). Autoradiographic characterization of neurotensin receptors in the entorhinal cortex of schizophrenic patients and control subjects. *J Neural Transm Gen Sect* **102**, 55-65.

Wong, W. T., Schumacher, C., Salcini, A. E., Romano, A., Castagnino, P., Pelicci, P. G. & Di Fiore, P. (1995). A protein-binding domain, EH, identified in the receptor tyrosine kinase substrate Eps15 and conserved in evolution. *Proc Natl Acad Sci U S A* **92**, 9530-4.

Wood, J. G., Hoang, H. D., Bussjaeger, L. J. & Solomon, T. E. (1988). Neurotensin stimulates growth of small intestine in rats. *Am J Physiol* **255**, G813-7.

Wood, S. M., Wood, J. R., Ghatei, M. A., Lee, Y. C., O'Shaughnessy, D. & Bloom, S. R. (1981). Bombesin, somatostatin and neurotensin-like immunoreactivity in bronchial carcinoma. *J Clin Endocrinol Metab* **53**, 1310-2.

Wu, X., Zhao, X., Baylor, L., Kaushal, S., Eisenberg, E. & Greene, L. E. (2001). Clathrin exchange during clathrin-mediated endocytosis. *J Cell Biol* **155**, 291-300.

Yamabhai, M., Hoffman, N. G., Hardison, N. L., McPherson, P. S., Castagnoli, L., Cesareni, G. & Kay, B. K. (1998). Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J Biol Chem* **273**, 31401-7.

Yamada, E. (1955). The fine structure of the gallbladder epithelium of the mouse. *Journal of Biophysical and Biochemical Cytology* **1**, 445 - 458.

Yamada, M., Ohata, H., Momose, K. & Richelson, E. (1995). Pharmacological characterization of SR 48692 sensitive neurotensin receptor in human pancreatic cancer cells, MIA PaCa-2. *Res Commun Mol Pathol Pharmacol* **90**, 37-47.

Zelhof, A. C., Bao, H., Hardy, R. W., Razzaq, A., Zhang, B. & Doe, C. Q. (2001). Drosophila Amphiphysin is implicated in protein localization and membrane morphogenesis but not in synaptic vesicle endocytosis. *Development* **128**, 5005-15.

Zeytinoglu, F. N., Gagel, R. F., Tashjian, A. H., Jr., Hammer, R. A. & Leeman, S. E. (1980). Characterization of neurotensin production by a line of rat medullary thyroid carcinoma cells. *Proc Natl Acad Sci U S A* **77**, 3741-5.

Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G. & Ferguson, S. S. (1999). Cellular trafficking of G protein-coupled receptor/beta-arrestin endocytic complexes. *J Biol Chem* **274**, 10999-1006.

Zhang, J., Ferguson, S. S., Barak, L. S., Menard, L. & Caron, M. G. (1996). Dynamin and beta-arrestin reveal distinct mechanisms for G protein- coupled receptor internalization. *J Biol Chem* **271**, 18302-5.