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Emerging concepts of receptor endocytosis and concurrent intracellular signaling: Mechanisms of guanylyl cyclase/ natriuretic peptide receptor-A activation and trafficking

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Abstract

Endocytosis is a prominent clathrin-mediated mechanism for concentrated uptake and internalization of ligand-receptor complexes, also known as cargo. Internalization of cargo is the fundamental mechanism for receptor-dependent regulation of cell membrane function, intracellular signal transduction, and neurotransmission, as well as other biological and physiological activities. However, the intrinsic mechanisms of receptor endocytosis and contemporaneous intracellular signaling are not well understood. We review emerging concepts of receptor endocytosis with concurrent intracellular signaling, using a typical example of guanylyl cyclase/natriuretic peptide receptor-A (NPRA) internalization, subcellular trafficking, and simultaneous generation of second-messenger cGMP and signaling in intact cells. We highlight the role of short-signal motifs located in the carboxyl-terminal regions of membrane receptors during their internalization and subsequent receptor trafficking in organelles that are not traditionally studied in this context, including nuclei and mitochondria. This review sheds light on the importance of future investigations of receptor endocytosis and trafficking in live cells and intact animals *in vivo* in physiological context.

Keywords

Membrane receptors; internalization; intracellular signaling; subcellular trafficking; short-signal motifs; guanylyl cyclase/natriuretic peptide receptor

1. Introduction

There are at least two types of cardiac-derived peptide hormones, namely atrial and brain natriuretic factor or peptides (ANF/ANP, BNP), which mediate natriuretic, diuretic, vasorelaxant, and antimitogenic responses directed to reduce blood pressure and maintain fluid volume homeostasis [1–7]. Both ANP and BNP are primarily synthesized in the heart atrial myocytes and to a lesser extent in the ventricular cells [8–10]. Pro-ANP, but not ANP, is stored in the atrial dense granules [11–13]. ANP is largely released as a 28-amino acid

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residues biologically active mature hormone, whereas BNP is released as a prohormone (Pro-BNP). Upon secretion, Pro-BNP is enzymatically cleaved to produce biologically active 32-residues BNP [11–13]. It is unclear whether BNP or Pro-BNP is stored in those granules, which contain ANP. A third peptide in the natriuretic peptide hormone family, differs from ANP and BNP in the tissue expression and biological function, is known as C-Type natriuretic peptide (CNP), which is highly conserved among species, is basically present in endothelial cells and the central nervous system [14, 15]. The receptors of these peptide hormones have been classified as natriuretic peptide receptor-A (NPRA), -B (NPRB), -C (NPRC), and ANF-RGC [16–24]. NPRA and NPRB show similar homology and contain an extracellular ligand-binding domain, a single transmembrane region, and an intracellular region containing both a protein kinase-like homology domain (protein-KHD) and guanylyl cyclase (GC) catalytic domain [16, 17, 20, 23–25]. As shown in Figure 1, both ANP and BNP specifically bind and activate NPRA that produces intracellular second-messenger cGMP in response to hormone binding in various cells and tissues [2, 26–29]. BNP is also considered as a neuropeptide hormone, which is expressed in a subset of transient receptor potential vanilloid-1 (TRPV1) neurons. A study showed that *Nppb*$^{-/-}$ mice selectively lost almost all behavioral responses to itch-inducing agents [30]. CNP activates NPRB that also produces intracellular cGMP [17, 24, 28, 29]. Moreover, all three natriuretic peptides arbitrarily bind to NPRC, which lacks a GC domain and does not produce cGMP [5, 22, 28, 31]. GC-A/NPRA is considered to be the biologically active cognate receptor of both ANP and BNP. Because most of the physiological effects of GC-A/NPRA are triggered by generating second-messenger cGMP, this receptor is generally considered the biologically active cognate receptors of ANP and BNP [5, 19, 29, 32–35].

The biological function of NPRA is demonstrated primarily through the ANP/BNP-dependent GC catalytic activity of the receptor and the production of cGMP, which is regulated by several factors, including hormones, growth factors, physiological milieu, and the ligand itself [26, 34, 36–42]. Several of our studies have established that the gene targeting (gene-disruption and gene-duplication) of *Npr1* (encoding NPRA) in mouse models shows the hallmark significance of NPRA signaling in lowering arterial pressure and protecting against renal, cardiac, and vascular diseases [43–48]. More recent evidences suggest that reduced levels of ANP seem to be associated with insulin resistance, obesity, lifestyle-associated metabolic syndrome, and essential hypertension [10, 49–51]. In addition, ANP/NPRA system has been suggested to play role in vascular remodeling of uterine spiral artery [52]. Previous studies have suggested that endothelial action of ANP enhances the myocardial inflammatory infiltration in early phase after acute infarction [53]. Further, ANP has been shown to exhibit anti-inflammatory properties and tumor-associated immune response [54, 55]. Moreover, the synthetic novel designer natriuretic peptides have provided the renal enhancing actions of these hormones over the naturally occurring molecules [56–59].

NPRA is designated as the cognate receptor for both ANP and BNP; nevertheless, ANP binds to the receptor with a higher affinity than BNP [60]. However, it is not yet clear whether ANP and BNP complete each other for the receptor binding. The ligand binding of both NPRA and NPRB has demonstrated that bound ligand-receptor complexes of NPRA are promptly internalized into cells, redistributed into intracellular compartments, and

ultimately degraded in lysosomes [35, 40, 61–64]. Ligand-receptor complexes of ANP-NPRA are distributed, possibly through the endosomes, to lysosomal compartments, where they are largely metabolized; however, a population of ligand-receptor complexes escapes the lysosomal compartments, allowing these receptors to recycle back to the plasma membrane [26, 34, 42, 62]. Moreover, internalization of ANP, BNP, and CNP also occurs through NPRC [65, 66]. Virtually, the ligand-mediated receptor endocytosis is a vital mechanism for physiological responses. Clathrin-mediated endocytosis is a well-established mechanism for numerous membrane-bound hormone receptors containing glucagon, insulin, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), which are also internalized by ligand-mediated endocytosis [35, 67, 68]. Our recent studies have demonstrated that NPRA is dynamin-dependently endocytosed in clathrin-coated vesicles [69]. Here, we provide a comprehensive summary of past and present findings on the internalization, intracellular trafficking, and concurrent signaling mechanisms of NPRA focused on cell-based studies as well as in intact live cells and animals *in vivo* to establish the actions of NPRA in the dynamic physiological context.

2. Historical background

Several studies have clearly established that ANP-BNP/NPRA ligand-receptor complexes are internalized in various cell types upon stimulation by ligand. However, the question of whether NPRA is internalized remained open for a long time. For example, earlier it has been stated that neither NPRA nor NPRB ligand-receptor complexes are internalized and that only NPRC receptor is internalized to downregulate the cGMP signaling process [70]. Although remarkable progress has been made toward determining the structure-function relationship of NPRA and NPRB, the issues of internalization and the trafficking itinerary of these GC receptors remained controversial until recently. In the past, debate focused on whether ANP/NPRA complexes were internalized at all or whether cells used some other mechanisms to cell-specifically release ANP from its receptor. Indeed, it was reported by default that among the three natriuretic peptide receptors, only NPRC was internalized with bound ligand [71]. It was suggested that endogenous NPRA was not internalized in cultured renal medullary interstitial cells and that rapid dissociation of ligand-receptor complexes occurred after ANP binding to NPRA at 37^0C. However, it was difficult to interpret the results of such findings because the dissociation of ligand was carried out in a medium containing high concentrations of unlabeled ANP to preclude the rebinding of dissociated hormone to receptors [71]. One study using this ligand-binding protocol indicated that intact ^{125}I-ANP was released into the culture medium of 293 T cells expressing recombinant NPRA [72]. However, in a later study, the authors reconciled the absence of ANP/NPRA internalization in 293 T cells, stating that it might have resulted from the slow rate of cell-specific ANP degradation in these cells [73].

Multiple studies from our laboratory and others, which have used stoichiometric kinetic analyses of ANP/NPRA, have provided strong evidence that bound ligand-receptor complexes are internalized and processed intracellularly, and that degraded products are released into the culture medium of various cell types harboring endogenous receptors [34, 61, 63, 64, 74], The same events have been demonstrated in transfected COS-7 and HEK-293 cells expressing recombinant NPRA [62, 75, 76]. Moreover, in response to CNP-

binding, NPRB has been shown to be internalized and recycled back to the cell surface in cultured glioma cells and hippocampus neurons [77]. The prevailing consensus now is that, in response to ligand binding, both NPRA and NPRB are internalized into the cell interior and redistributed in subcellular compartments. To obtain unequivocal evidence, it became important to visualize cell trafficking and redistribution of ANP/NPRA complexes in intact cells. Recent findings from our laboratory have resolved this important issue, unequivocally demonstrating ligand-dependent endocytosis and intracellular trafficking of NPRA by confocal immunofluorescence (IF) and co-immunoprecipitation (Co-IP) analyses with organelle-specific marker proteins in eGFP/NPRA-transfected HEK-293 cells and primary MMCs [26, 42]. Our findings have facilitated direct visualization of ligand-dependent endocytosis, trafficking, and redistribution of eGFP-NPRA into subcellular compartments with concurrent production of intracellular second-messenger cGMP. Earlier we have also demonstrated the kinetics of ^{125}I-ANP-NPRA internalization in recombinant Cos-7 and HEK-293 cells [34, 75]. However, in recent studies, we examined immunofluorescence-based NPRA internalization in MMCs, a cell system with physiological relevance [26]. We used eGFP-NPRA to capture immunofluorescence images generated by this receptor protein only during the processes of endocytosis and trafficking, without any interference from either NPRB or NPRC. Also, since ANP binds to both NPRA and NPRC, we have used c-ANF to block ANP binding to NPRC in the ^{125}I-ANP binding assay. The peptide c-ANF binds only to NPRC, not to either NPRA nor NPRB. Thus, all the binding parameter for ligand-dependent internalization of ligand-receptor complexes were accounted due to NPRA as previously reported [78].

The mechanisms of NPRA signaling during the glycosylation of NPRA is not well understood. Previously, it was suggested that glycosylation sites in guanylyl cyclase-coupled receptors might be important for proper folding and stability of the receptor protein, as well as essential to facilitate ligand binding of NPRA and NPRB [18, 79, 80]. However, one study has suggested that glycosylation is not essential for ligand-binding of NPRA [81]. More experimentations are needed to confirm the functional role of glycosylation in the ligand binding and trafficking of both NPRA and NPRB GC-coupled receptors.

3. Ligand-stimulated internalization of NPRA

After ANP binding, NPRA dimerizes, while the GC catalytic domain of the receptor becomes activated [82]. Studies have shown that after ANP binding, ligand-receptor complexes are internalized, redistributed in the cell interior, and metabolized in lysosomes [40, 61, 63, 64, 74]. It has been suggested that internalized receptor traffic into intracellular compartments, as well as a population of receptor, escapes the lysosomal compartments and recycles back to plasma membranes [34, 35, 42, 61, 75, 83]. Our classical studies using Leydig tumor (MA-10) cells containing a high density of endogenous NPRA and recombinant human embryonic kidney-293 (HEK-293), as well as COS-7 cells transfected with NPRA cDNA construct, firmly established that ligand-receptor complexes of ANP-NPRA are rapidly and ligand-dependently internalized and redistributed intracellularly [61, 84, 85]. Those findings demonstrated that after ligand-binding NPRA is internalized with bound hormone by an energy- and temperature-dependent mechanism, after which degraded ligand is released into the culture medium [34, 42, 61, 62, 86].

Distribution of ^{125}Iodine-labeled-ANP (^{125}I-ANP) radioactivity on the cell surface, in the intracellular compartments, and in culture medium established a dynamic equilibrium of receptor-mediated ^{125}I-ANP uptake, degradation, and release outside of cells (Fig. 2). A major proportion of internalized ^{125}I-ANP was released into the culture medium, which consisted of approximately 75%-80% degraded products and about 20%-30% intact ligand [26, 34, 42, 61, 62]. The release of both degraded and intact ligands was prevented by introducing the lysosomotropic agents such as ammonium chloride (NH_4Cl_2), chloroquine, or nigericin in the culture media [40, 42, 61]. Early work suggested that most of the internalized ^{125}I-ANP was metabolized through the pathways of lysosomal degradative compartments in intact cells; however, a population of internalized ligand-receptor complexes recycled back to the plasma membrane and ligand was extruded outside the cell (Fig. 3). It is believed that the released intact ligand could rebind to the receptor and re-enter the cell by the pathway of the retro-endocytosis mechanism [35, 61, 84].

Although, after internalization, most of the endocytosed ligand was degraded in the lysosomes and then released into the culture medium, a population of ligand-receptor complexes escaped the lysosomal degradative pathway and extruded intact outside of cells [34, 62, 87]. By using an antibody-tracking method, one study indicated that, in a cell-specific manner, both NPRA and NPRB are ligand-independently internalized [70]. However, antibody-tracking could determine the internalization kinetics of ligand-receptor complexes only qualitatively. Our recent studies using confocal immunofluorescence microscopy clearly visualized and unequivocally established that ligand-dependent internalization and subcellular trafficking of eGFP-tagged-NPRA (eGFP-NPRA) occurs in stably transfected recombinant HEK-293 cells and transiently NPRA-transfected primary murine mesangial cells (MMCs) [26, 42]. Furthermore, NPRB has been shown, in response to CNP binding, to be internalized and recycled back to the cell surface in cultured hippocampal neurons and C6 glioma cells [77].

4. Role of clathrin adaptor proteins in the endocytosis of NPRA

It is thought that the tyrosine-based Yxxphi sorting signals located in the carboxyl-terminus domain direct the internalization and trafficking of various membrane receptors by interacting with mu 1, mu 2, mu 3, and mu 4 subunits of adaptor proteins (APs), including, respectively, AP-1, AP-2, AP-3, and AP-4 [26, 88, 89]. Recent studies have demonstrated the role of adaptor protein 2 in endocytosis of insulin-like growth factor-I receptor (IGF-IR), which interacts with insulin-receptor substrate (IRS)-1 with the clathrin adaptor complex AP2 [90]. However, IRS-1 inhibits recruitment of IGF-IR into clathrin-coated structures; for this reason, IGF-IR avoids rapid endocytosis and prolongs its activity on the cell surface. Beta-arrestins are known to act as endocytic adaptors by recruiting the AP-2 complex to G-protein-coupled receptors (GPCRs), linking them to clathrin-coated pits (CCPs) for internalization [91]. Upon activation by Wnt, the Frizzled receptor is internalized in a process that requires the recruitment of Dishevelled, a family of proteins involved in the Wnt signaling pathways [92]. Interaction between Dishevelled2 (Dvl2) and μ2-adaptin, a subunit of the clathrin adaptor AP-2, is required to engage activated Frizzled4 with the endocytic machinery for its internalization.

Our recent studies have shown that the μ1B subunit of AP-1 directly binds to a phenylalanine-based FQQI motif in the cytoplasmic tail of NPRA receptor protein [26]. However, more studies are needed to establish the exact role of adapter proteins in the endocytotic pathways of NPRA. To delineate the critical role of endocytic signals in the intracellular sorting and signaling of NPRA, immunofluorescence staining and Co-IP of eGFP-NPRA with the AP-1 marker have been used to follow the intracellular trafficking and concurrent signaling of mutated and WT receptors by confocal immunofluorescence microscopy and immunoblotting in MMCs (Fig. 3). Subcellular trafficking of receptor has shown that the immunofluorescence colocalization of mutated receptors with μ1B markers was decreased by 50% for the subunit of AP-1 as compared with the WT receptor in intact MMCs [26]. It has been shown that the autosomal recessive hypercholesterolemia protein (ARH) is important in clathrin-mediated endocytosis of low-density lipoprotein receptors (LDLRs) [93]. In addition to its role in endocytosis, ARH unites with AP-1B in basolateral exocytosis of LDLR from recycling endosomes (REs) to plasma membrane.

5. Role of dynamin in NPRA endocytosis

In recent studies using ^{125}I-ANP binding assay and confocal microscopy, we have examined the role of dynamin in the internalization and trafficking of NPRA in a stably transfected HEK-293 cells [69]. Our findings indicated that ANP treatment of high-density NPRA expressing recombinant HEK-293 cells time-dependently accelerates internalization of receptor from the plasma membrane to the cell interior. However, the internalization of ligand-receptor complexes of NPRA was significantly decreased by specific inhibitors of clathrin- and dynamin-dependent receptor internalization. These decreases were approximately 80%-90% using monodansylcadaverine (MDC), 75%-80% using chlorpromazine (CPZ), and 85%-90% using mutant dynamin, which are specific blockers of endocytic vesicle formation and internalization pathways (Fig. 4). Moreover, IF visualization of the internalization of NPRA and enhanced GFP-tagged NPRA in HEK-293 cells by confocal microscopy have demonstrated the formation of endocytic vesicles after 5 min of ANP treatment; this effect was blocked by clathrin inhibitors (MDC and CPZ) and mutant dynamin construct [69]. These studies showed that NPRA undergoes internalization via clathrin-mediated endocytosis as part of its normal cellular itinerary, including receptor trafficking, signaling, and metabolic degradation.

Our earlier findings suggested that ANP-mediated internalization of NPRA in the recombinant HEK-293 cells occurred via the clathrin-dependent pathway and involved initial clustering of receptor-ligand cargo in clathrin-coated pits, with plasma membrane invagination forming clathrin-coated vesicles. Blockade of ligand-induced endocytosis of the receptor by MDC and CPZ is the most effective means of the mechanistic action that inhibits the receptor internalization. Confocal microscopy clearly showed the formation of endocytic vesicles, which is a characteristic feature of internalized receptor within the cytoplasm. Several previous studies have shown that both CPZ and MDC, as well as mutant dynamin, have effectively blocked endocytosis of the membrane receptors and affected both the assembly of clathrin components and the formation of clathrin-coated pits in cells of various types [94–98]. The classical clathrin-mediated endocytic pathway clearly seems to be a major route for the endocytosis of NPRA. Demonstration of this provides a significant

advance in our understanding of the role of this receptor in regulating hypertension and cardiovascular homeostasis.

In recent studies, we have found that MDC and CPZ effectively blocked the internalization of ANP-NPRA complexes in recombinant HEK-293 cells by approximately 80%-90% [69]. MDC acts as a competitive blocker of transglutaminase by creating an isopeptide bond between the glutamine and lysine amino-acid residues of two proteins, thus inhibiting the clathrin-coated vesicle-dependent endocytic process [96, 99, 100]. Earlier findings suggested that MDC blocks the internalization and trafficking of various types of ligand-receptor complexes [101–103]. Similarly, CPZ has been shown to block the assembly of clathrin-coated pits and to inhibit coated-vesicle-mediated internalization of membrane proteins [104–106]. We demonstrated that CPZ inhibits endocytosis of ANP-NPRA complexes by 80%-85%, which is consistent with the previous finding that CPZ inhibits the endocytosis of several different types of hormone receptors [106–109]. CPZ, MDC, and dynamin-mutant have also been shown to play inhibitory roles for endocytosis in Wnt signaling [110].

The expression of a dominant-negative mutant of dynamin (K44A/dynamin) has been shown to block the formation of coated vesicles during the internalization of various ligand-receptor complexes [67, 111–113]. Previous studies have also indicated that dynamin, through its interaction with AP2, has a critical role in the localization of clathrin-coated pits and clathrin-mediated endocytosis [114, 115]. After recruitment to coated pits, dynamin forms a ring structure, and then pinches off coated vesicles into the cytoplasm. This process, known as pinchase, through its intrinsic GTPase activity, generates clathrin-coated vesicles in the clathrin-mediated endocytosis [105, 116–118]. It is thought that the GTPase-defective dominant mutant interferes with wild-type dynamin and blocks the formation of endocytic coated-vesicles and internalization of membrane receptors [119–123].

The endocytic inhibitors MDC and CPZ and dynamin GTPase blocker (dynamin mutant) have been shown to significantly decrease the intracellular accumulation of cGMP. Moreover, earlier reports suggested that G-protein-coupled receptor (GPCRs) continue to signal by generating cAMP throughout internalization processes with their bound ligands [124]. Signaling from inside the cell is persistent and appears to trigger specific downstream effects. We have recently demonstrated that intracellular accumulation of cGMP significantly decreases after treatment with endocytic inhibitors MDC and CPZ and transfection with dynamin mutant GTPase blocker [69]. Diminution in the intracellular accumulation of cGMP indicates that these same endocytic inhibitors and dynamin GTPase blocker (dynamin mutant) have a concerted regulatory role in NPRA trafficking and signaling process.

6. Post-endocytic sorting of NPRA

Recently we visualized the internalization and subcellular trafficking of enhanced GFP (eGFP)-tagged NPRA (eGFP–NPRA) in intact recombinant HEK-293 cells and primary MMCs, using immunofluorescence and co-IP analyses of eGFP–NPRA [26, 42]. These studies showed that treatment of cells with ANP initiated rapid internalization and co-localization of the receptor with early endosome antigen-1 (EEA-1), which was increased in

a time-dependent manner. The highest visualization, which occurred at 5 min, gradually decreased within 30 min. Similarly, co-localization of the receptor was time-dependently accomplished with lysosome-associated membrane protein-1 (LAMP-1) in MMCs [26]. These immunofluorescence and co-IP studies demonstrated that after trafficking in the endosomes, eGFP-NPRA routed intracellularly within the late endosomes and/or lysosomes. The accumulation of eGFP–NPRA in intracellular compartments was observed after treatment of MMCs with lysosomotropic agents such as chloroquine and NH_4Cl_2. In ANP-treated cells, eGFP–NPRA was time-dependently co-localized with LAMP-1; however, after treatment with lysosomotropic agents, intracellular accumulation of the receptor gradually increased within 30 min. It is known that both chloroquine and NH_4Cl_2 inhibit lysosomal degradation of intracellular trafficking of the various ligand-receptor complexes [34, 35, 40, 125]. Moreover, lysosomotropic agents did not completely block the receptor degradation of ANP/NPRA complexes in lysosomes, suggesting that the trafficking of intact eGFP–NPRA also occurred through a lysosome-independent pathway. In our studies, co-IP assays confirmed that the localization of internalized NPRA occurred within subcellular organelles during endocytosis of eGFP-NPRA [42]. The endocytic recycling marker Rab 11, which was used as a recycling endosome, showed that ~20 % of receptors recycled back to the plasma membrane [26, 42]. Our results support the notion that ANP-treated cells exhibit a marked increase in the IF of cGMP, whereas receptor still trafficked into intracellular compartments. This suggests that after ligand binding, NPRA is rapidly internalized and trafficked from the cell surface into endosomes, including recycling endosomes, late endosomes, and lysosomes, with concurrent generation of intracellular second-messenger cGMP.

On the other hand, subcellular trafficking of NPRA indicated that IF of the mutated receptor with EEA-1, LAMP-1, and Rab 11 markers was decreased by almost 50%-58% in early endosomes, 45%-50% in lysosomes, and 35%-45% in recycling endosomes, respectively, compared with WT NPRA in MMCs and HEK-293 cells [26, 42]. To support the findings of immunofluorescence-based internalization kinetics and cell trafficking of WT and mutated eGFP-NPRA in intact MMCs, we have used the ^{125}I-ANP binding assay to examine cell-surface and intracellular ligand-receptor complexes [26]. These studies demonstrated that ^{125}I-ANP binds to cell-surface eGFP-NPRA, enters through the process of receptor-mediated endocytosis, and then is delivered to intracellular compartments until it reaches a steady-state level, which occurs after 15-30 min. The kinetic rates of internalization, degradation, and release of ^{125}I-ANP were markedly decreased in the mutant receptor, suggesting that the FQQI motif is important for the internalization and subcellular trafficking itinerary of eGFP-NPRA in intact cells [26]. Thus, it seems that homeostatic regulation of native NPRA and the cell sensitivity of ANP depends on a dynamic equilibrium and reuse of ligand-receptor complexes from the cell surface to intracellular compartments.

Moreover, we have demonstrated that internalization and subcellular trafficking of NPRA, using IF staining (IFS) and co-IP of plasma membrane, endosomal, lysosomal, and recycling endosome markers to follow intracellular trafficking and signaling by confocal IF microscopy (CIF) and immunoblotting (IB) analyses in recombinant HEK-293 cells [42]. These functional subcellular compartments provide the physical basis for the assembly and turnover of intracellular kinetics of ligand-receptor complexes, which in turn define the

specialized endosomal–lysosomal signaling platforms for hormone-dependent physiological functions of membrane receptors and other membrane proteins.

7. Role of short-signal motif in the internalization and trafficking of NPRA:

Co-immunoprecipitation assays confirmed that colocalization of the mutant receptor (FQQI/AAAA) was significantly decreased in subcellular compartments during endocytosis and trafficking processes [26]. These earlier studies showed that the FQQI signal sequence motif is critically essential for the internalization and subcellular trafficking of NPRA during the hormone signaling process in intact primary MMCs [26]. The confocal immunofluorescence studies also showed that, after ligand binding, WT receptor eGFP-NPRA is rapidly internalized and trafficked from the cell surface to subcellular compartments. However, the mutated motif FQQI/AAAA significantly inhibited internalization, subcellular trafficking, and signaling processes of NPRA receptor protein [26]. These studies also demonstrated that FQQI has a significant role in sustaining receptor signaling in intact MMCs and thus advanced our understanding of the molecular mechanisms of vesicular transport, subcellular trafficking, and concurrent NPRA signaling. Similarly, another mutation in the GDAY motif (GDAY/AAAA) in the NPRA carboxyl-terminal domain has been shown to reduce internalization of mutant receptors by almost 40%-45% as compared with the WT receptor [62].

Small signal-sequence motifs have been shown to play pivotal roles in endocytosis and intracellular trafficking of membrane receptor proteins to direct cargo into trafficking vesicles [126, 127]. Short signal sequence motifs constitute the short sequence of amino acids in a linear array that consists of two-to-six amino-acid residues. Nevertheless, only two or three residues have critical roles in receptor endocytosis and trafficking pathways [126, 128, 129]. The most common small-signal sequence motifs found in various membrane receptors are shown in Table 1. Tetrameric sequence Gly^{920}-Asp^{921}-Ala^{922}-Tyr^{923} (GDAY) motif in the carboxyl terminal-region of NPRA serves as a signal for endocytosis [62]. The amino acid residues Gly^{920} and Tyr^{923} constitute the critical elements for internalization of NPRA in the GDAY signal motif, however, Asp^{921} exhibits an acidic environment for efficient signaling of GDAY during the subcellular trafficking process. The mutation of Gly^{920} and Tyr^{923} to Ala prevented the internalization of NPRA by almost 50%, but had no effect on the recycling process. Although, the mutation of Asp^{921} to Ala did not exhibit a major effect on receptor endocytosis, but critically attenuated the recycling of internalized receptors to the plasma membrane.

The NPxY signal sequence is known to facilitate the internalization of low density lipoprotein (LDL) receptor [130]. Endocytosis of platelet-activating factor is governed by a putative DPxxY signal motif and type-2 vasopressin receptor by NPxxY motif [131, 132]. Similarly, YxxL signal motif also functions in endocytosis of LDL receptor-related protein [133]. A common feature of these internalization signal motifs, including DPxxy, NPxY, and GDAY, is the presence of an amino acid tyrosine at the end of the tetrapeptide short sequence motif [62, 131]. Tyrosine residues are also involved in endocytosis of mannose-6-phosphate receptor and in the influenza virus hemagglutinin, even though they are not present in the context of NPxY or YxRF consensus sequence suggesting that if a universal

internalization signal exists, it may not contain a universal peptide sequence [131, 134]. The critical characteristics of all these sequences might be their specification of a particular conformation, such as a tight beta-turn in protein structure [135]. Tyr recognition short sequence signal motifs present a small surface loop but they differ in primary structure in the context of the positioning of Tyr residue in the loop structure [128, 136, 137]. The substitution of Tyr with amino acid known to be inert in endocytosis resulted in disruption of the beta-turn structure. The tyrosine-based motifs such as NPxY and YxxL recruit clathrin and adaptor protein molecules and act as a ligand-receptor cargo recognition sequence for their delivery to endosomes and lysosomes [134, 138]. These motifs initially recruit AP-2 at the plasma membrane and activate mu2 subunit that facilitates the binding of beta-2 subunit of AP-2 to clathrin at the cell surface, leading to clathrin-mediated internalization of receptor molecules [128, 134, 139, 140].

NPxY was recognized as the first short signal sequence motif in the cytoplasmic domain of membrane receptors and proteins with critical roles in internalization and trafficking of receptor molecules [130]. Including LDL receptor, beta-1 integrin, megalin, beta-amyloid precursor protein, EGF receptor, and neurotrophin receptor [93, 128, 130, 141–144]. The early studies demonstrated that substitution of a cysteine residue for a tyrosine residue in NPxY (Asn-Pro-x-Tyr) motif of LDL receptor rapidly abolished its endocytosis [145]. Several dileucine-based motifs, including YGLL, SLL, and YWLL are also located in the protein-KHD and GC regions of NPRA, however, their roles in the internalization and trafficking of NPRA is not yet known. Dileucine (LL) motifs regulate internalization and trafficking of several membrane receptors and proteins through the endocytic and secretory pathways [128, 146–150]. LL motifs contain 3-7 amino acid residues in which LL amino acid residues are preceded by a polar amino acids and a negatively charged residue that may be aspartic acid, glutamic acid, or phosphoserine. Dileucine motifs with acidic amino acids are constitutively active and regulate both endocytosis and secretory pathways of membrane receptors and proteins [148].

A short sequence motif Yxxphi is located in the carboxyl-terminal domain of NPRA at residues Y^{988}-x-x-F^{991}; however, its role in the internalization of NPRA remains be determined. These signal motifs with internalization specificity are located within 10-30 amino acid residues from the transmembrane domain of membrane receptors [151–153]. The tyrosine-based Yxxphi sorting signals direct the internalization by interacting with mu1, mu2, mu3, and mu4 subunits of adaptor proteins, including AP-1, AP-2, AP-3, and AP-4, respectively [88, 89, 139]. The tetrapeptide sequence Yxxphi is usually located in the cytoplasmic domains of the receptors including transferrin and asialoglycoprotein receptors and exhibit critical roles in the endocytosis. In Yxxphi signal motifs, Y represents a tyrosine residue, x is any amino acid residue, and phi represents a residue with large bulky hydrophobic side-chain. Yxxphi signal motifs contain dual specificity with endocytotic functional motif and a trafficking signal within the endosomal and/or secretory pathways [128, 134, 153, 154]. The adaptor protein complexes capture the cargo in the coated vesicles for trafficking of receptors in the subcellular compartments.

8. Receptor internalization in non-traditional sub-cellular compartments:

Here, we predict that the alternative fates of activated NPRA are emerging, including trafficking to two other subcellular compartments such as the nucleus and mitochondria (Fig. 5). Comprehensive investigations of receptor trafficking have demonstrated that growth factors induce the internalization of receptor tyrosine kinases (RTKs) and then are either sorted to lysosomes or recycled back on plasma membranes [155–157]. In addition, RTKs traffic through different subcellular compartments such as the nucleus and mitochondria. It has been recognized that trafficking to these novel destinations involves newly identified biochemical and cellular mechanisms and that these trafficking events function in the signal transduction pathways, implicating the receptor itself as a signaling element between the cell surface and the subcellular organelles [158, 159]. Similarly, after binding of ligand, cell-surface epidermal growth factor receptor (EGFR) is internalized and trafficked through the endo-lysosomal subcellular compartments or returned to the plasma membrane by recycling mechanisms. Other than these subcellular compartments, EGFR also traffics from the cell surface to the nucleus, Golgi apparatus, endoplasmic reticulum, and mitochondria after endocytosis, where it seems to be involved in multiple biological and physiological functions [160].

Evidence suggest that angiotensin (Ang II) type 1 receptor (AT1R) levels are greatly increased in the mitochondria of aged mice, however, AT2R was localized in mitochondria at a greatest density in young animals and decreased with age [161, 162]. Those previous studies indicated that Ang II and AT2R constitute essential components of the mitochondrial inner membrane of various cell types and regulates nitric oxide synthesis to enhance mitochondrial function. These observations also support the hypothesis of intracrine Ang II as previously suggested to illustrate the well –established physiological system [163]. The mechanisms by which these receptors are translocated into the inner membrane of the mitochondria remain unknown. Usually several receptors and proteins localized in mitochondria, are synthesized in the cytoplasm and contain mitochondrial localization sequence at the amino-terminal, which are transported with the help of cytosolic chaperons and delivered to the inner and outer membranes of mitochondria [164, 165]. Interestingly, alternative pathways have been suggested for internalization of Ang II ligand-receptor complexes into the subcellular compartments, including microtubule-dependent endocytic pathway [166]. Recent studies have indicated that luminal receptor-bound Ang II is endocytosed in a complex of both AT1R and AT2R as a heterodimer in the endoplasmic reticulum in cultured LLC-PK1 cells [167]. More studies are needed to determine the diversified pathways of the internalization and trafficking of various ligand-receptor complexes in diverse subcellular compartments in both physiological and pathophysiological contexts.

9. Intracellular trafficking with concurrent signaling of NPRA

Our recent studies have shown internalization and concurrent signaling of NPRA in subcellular compartments; this had not been previously demonstrated [26, 42]. Preparation of the enhanced GFP (eGFP)-tagged NPRA (eGFP-NPRA) construct has greatly helped to visualize the internalization, intracellular trafficking, and subsequent signaling of receptor in

the subcellular compartments. These studies delineated the molecular mechanisms of NPRA trafficking with ANP/NPRA/cGMP signaling in recombinant HEK-293 cells and primary MMCs in the physiological context [26, 42]. Earlier studies showed that cGMP production is not exclusively activated at the cell surface, but also occurs after receptors have been internalized and continued trafficking in subcellular locations [26, 42]. However, endocytic inhibitors MDC and CPZ and dynamin GTPase blocker (dynamin mutant) significantly decrease the intracellular accumulation of cGMP [69]. Moreover, the receptor containing the mutated short signal motif (FQQI/AAAA) also produced a significantly lower level of intracellular cGMP during subcellular trafficking than do the WT receptor without this mutation [26].

Interestingly, early studies reported that G-protein-coupled receptor (GPCR) continued, using their bound ligands, to produce signals by generating intracellular second-messenger cAMP throughout the internalization and trafficking itinerary [124]. Moreover, cGMP regulates various processes, including hypertension, cardiovascular homoeostasis, antimitogenic effects vascular reactivity, sensory transduction, anti-inflammation responses, neuronal plasticity, and learning [124, 168]. Generally, cGMP interacts with three distinct kinds of intracellular effector protein molecules, including cGMP-dependent protein kinases (PKG), cGMP-activated phosphodiesterases (PDEs), and cGMP-regulated ion channels (CNGs) [169]. It is possible that cGMP-binding proteins transduce the cGMP signal to alter cell function through different mechanisms, comprising an inhibitory effect and/or by stimulating protein phosphorylation [170, 171]. Using immunofluorescence microscopy of cGMP, it was possible to clearly visualize that ligand-receptor complexes of ANP/NPRA continuously produce intracellular cGMP at various time points after treatment with ANP during the internalization and trafficking of this receptor protein [26, 42, 69]. Based on our previous studies, it became evident that signaling from inside the cell into subcellular compartments is persistent. This signaling appears to trigger specific downstream effects of cGMP [26, 42]. Attenuation of the intracellular accumulation of cGMP suggested that endocytic inhibitors and dynamin GTPase blocker may have a concerted regulatory role in NPRA trafficking and signaling in intracellular compartments [69].

10. Live-cell and intact-animal *in vivo* approaches to internalization and trafficking of membrane receptors

Internalization and subcellular trafficking are critical for most membrane receptors with approaches in different subcellular compartments and some receptors that are recycled back to the plasma membrane during the signaling process. Live-cell imaging eliminates the possible effects of fixation on cell volume, as well as the loss reduction, or redistribution of GFP-chimeric receptors in physiological and pathophysiological contexts. Live-cell imaging permits investigation of receptor trafficking while avoiding confounding variables associated with fixation, particularly the loss or artifactual movement of eGFP-tagged membrane receptors. As individual cells are tracked in real time, the subcellular localization of receptors can be imaged and measured. Live-cell imaging allows the study of protein trafficking, migration, proliferation, apoptosis, cell-cycle pathway, autophagy, and protein-protein interactions and dynamics [172]. To understand the mechanistic pathway of receptor

endocytosis, trafficking, and signaling of live cells and animal *in vivo* have been used to determine the roles of various receptors in their physiological milieu.

Interestingly, *in vivo* infusion and uptake of Ang II in intact mice indicated that multiligand endocytic receptor megalin has at least some role in the uptake of Ang II and the downstream signaling process in proximal tubule cells (PTCs) *in vivo* [173]. Earlier *in vivo* studies showed that PTCs of the kidney also accumulate both systematically infused ^{125}I-labeled and unlabeled Ang II [174–176]. Live-cell imaging has also simultaneously captured time-lapse images of Ang II type 1a receptors (AT1aR) and intracellular compartments in transfected HEK-293 cells following stimulation with Ang II [172]. It has been reported that a proton-sensing GPCR, ovarian cancer G protein-coupled receptor 1 (OGR1), is related to acidosis and diseases that cause tissue acidification. pH-dependent intracellular trafficking of OGR1 has been visualized in living leukocytes by a real-time fluorescence microscopic method based on sortase A-mediated pulse labeling of OGR1. Quantitative single-cell image analysis and live-cell monitoring demonstrated that the signal transduction activity of OGR1 is regulated by pH-dependent receptor internalization and recycling to the plasma membrane [177]. Recently, internalization of G protein-coupled receptors (GPCRs) was visualized with high spatial resolution near the plasma membrane by means of total internal reflection fluorescence (TIRF) microscopy at a level of discrete single events. Methods have also been developed to determine the relative extent of internalized fluorescent receptor-ligand complexes by comparative fluorescence quantification in living cells of Chinese hamster ovaries (CHO) [178]. Live-cell imaging of other GPCRs using confocal microscopes enabled investigators to observe the internalization and trafficking of receptors in individual cells [179, 180].

Confocal imaging revealed detailed receptor neuroanatomy throughout the nervous system of knock-in mice, where δ-opioid receptor (DOR) was replaced by an active DOR-EGFP fusion protein [181]. Real-time imaging in primary neurons allowed dynamic visualization of drug-induced receptor trafficking. In DOR-EGFP animals, drug treatment triggered receptor endocytosis that correlated with the behavioral response. It also has been shown that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors play an important role in brain functions such as learning and memory process. Modulation of synaptic strength through trafficking of AMPA receptors is an essential mechanism underlying synaptic plasticity. Several studies have used live time-lapse imaging of fluorescence-tagged AMPA receptors to directly monitor membrane trafficking in these receptors in their basal state, as well as during synaptic plasticity *in vivo* using confocal microscopy [182]. Overexpression of lung 5-hydroxytryptamine7 (5-HT7) receptor is an important protective mechanism during lipopolysaccharide (LPS)-induced sepsis-related cell damage. 5-HT7 receptor trafficking has been evaluated using *in-vivo* and *in-vitro* models of LPS-induced inflammatory cell injury in rats and LPS-treated A549 cells, which suggested that lung 5-HT7 receptor expression is increased in LPS-induced endotoxemia [183]. Tumor hypoxia is linked with progression of malignant state and resistance to cancer treatments and therapies. It has been indicated that the ligand-bound EGF receptor (EGFR) trafficking is prolonged due to hypoxia-inducible factor (HIF)-medicated delays of endocytic cycle [184]. These authors suggested a role for oxygen-sensing pathway of tumor activation of HIF-associated EGFR-mediated signaling by delayed enocytic trafficking and deactivation of

receptor protein. To understand endocytosis, trafficking, and signaling of receptors, more studies with live-cell and animal *in-vivo* approaches will allow greater detail that should illustrate the mechanistic pathways of NPRA in both physiological and pathophysiological contexts of hormone signaling. The gene-knockout studies of *Npr1* in mice have demonstrated that ANP resistance is associated with sodium and volume retention and kidney dysfunction in the mutant animals [45]. Nevertheless, the role of NPRA trafficking in the recycling endosomes and the desensitization of this receptor under pathological conditions is not well understood.

11. Conclusions and future perspectives

Upon ligand-induced activation, NPRA is endocytosed and intracellularly sorted to endosomes, recycling endosomes, late endosomes, and finally to lysosomes. ANP binding to the receptor accelerates internalization and trafficking of ligand-receptor complexes of ANP/NPRA into intracellular compartments. Ligand is subsequently delivered through the endosomes to lysosomal compartments, where, in large measure, it is degraded. However, a population of ligand–receptor complexes escape and are diverted into recycling endosomes, allowing these receptors to recycle back to the plasma membrane. After exposure to ligand, the receptors, at various times during the subsequent internalization and subcellular trafficking processes, continuously produce the intracellular second messenger cGMP. Evidence suggests that intracellular accumulation of cGMP is significantly decreased after treatment with endocytic inhibitors MDC and CPZ and introduction of dynamin GTPase blocker (dynamin mutant), suggesting that dynamin has an intrinsic role in the endocytic process.

The review provides compelling evidence of the specificity of ligand-induced endocytosis of membrane receptors through clathrin-coated vesicle formation, as well as the role of dynamin GTPase in this process. Detailed investigation of endocytic proteins will be necessary to elucidate the precise functions of these proteins in receptor internalization and trafficking in intracellular compartments, as well as their subsequent signaling in physiological and pathophysiological conditions. Immunofluorescence studies have provided critical information regarding mechanisms of the trafficking itinerary of ANP-NPRA, during which it undergoes internalization via clathrin-mediated endocytosis and potentiates the development of pathophysiological conditions such as hypertension and cardiovascular disease. Exploitation of molecular and pharmacologic perturbants has facilitated delineation of the pathways of ligand-bound receptor endocytosis, trafficking, and redistribution in subcellular compartments from the plasma membrane into the cell interior. The demonstration of receptor trafficking in organelle that have not traditionally been studied, including nuclei and mitochondria, has opened a new line of investigation of receptor trafficking into subcellular compartments. Further concerted studies are needed to delineate receptor endocytosis and trafficking in their physiological contest in live cells and whole-animal models. Our understanding of the molecular determinants mediating the association of membrane receptors with subcellular organelles during intracellular trafficking with concurrent signaling should provide a basis for new molecular studies and important targets for therapeutic developments in translational health and disease.

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Abbreviations

APs	adaptor proteins
NH_4Cl_2	ammonium chloride
Ang II	angiotensin II
AT1R	angiotensin type 1 receptor
ANP	atrial natriuretic peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARH	autosomal recessive hypercholesterolemia
CNGs	cGMP-regulated gated ion channels
CPZ	chlorpromazine
CHO	Chinese hamster ovaries
CCPs	clathrin-coated pits
CIF	confocal IF microscopy
CNP	C-type natriuretic peptide
DOR	δ-opioid receptor
eGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
GPCRs	G-protein-coupled receptors
OGR1	ovarian cancer G protein-coupled receptor 1
GC-A/NPRA	Guanylyl cyclase/natriuretic peptide receptor-A
5-HT7	5-hydroxytryptamine7
HEK-293	Human embryonic kidney-293
IB	immunoblotting
IF	immunofluorescence
Co-IP	co-immunoprecipitation
IGF-IR	insulin-like growth factor-I receptor

IRS	insulin-receptor substrate
protein-KHD	protein Kinase-like homology domain
LDLR	Low-density lipoprotein receptor
MDC	monodansylcadaverine
MMCs	murine mesangial cells
NPRA	natriuretic peptide receptor-A
NPRB	natriuretic peptide receptor-B
NPRC	natriuretic peptide receptor-C
PDEs	phosphodiesterases
PDGF	platelet-derived growth factor
PKG	protein kinases G
PTCs	proximal tubule cells
REs	recycling endosomes
TIRF	total internal reflection fluorescence
TRPV1	transient receptor potential vanilloid-1

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Highlights

- Internalization is a prominent clathrin-mediated mechanism for concentrated uptake of ligand-receptor complexes for receptor-dependent regulation of cell membrane function, intracellular signal transduction, and physiological activities.
- The receptor endocytosis and contemporaneous intracellular signaling occur concurrently during internalization and subcellular trafficking with simultaneous generation of second-messenger signals in intact cells.
- The short-signal motifs located in the carboxyl-terminal regions of membrane receptors play pivotal roles during the internalization and subsequent receptor trafficking in subcellular organelles.
- Emphasis is placed on future investigations of receptor endocytosis and trafficking in live cells and intact animals *in vivo* in physiological context.

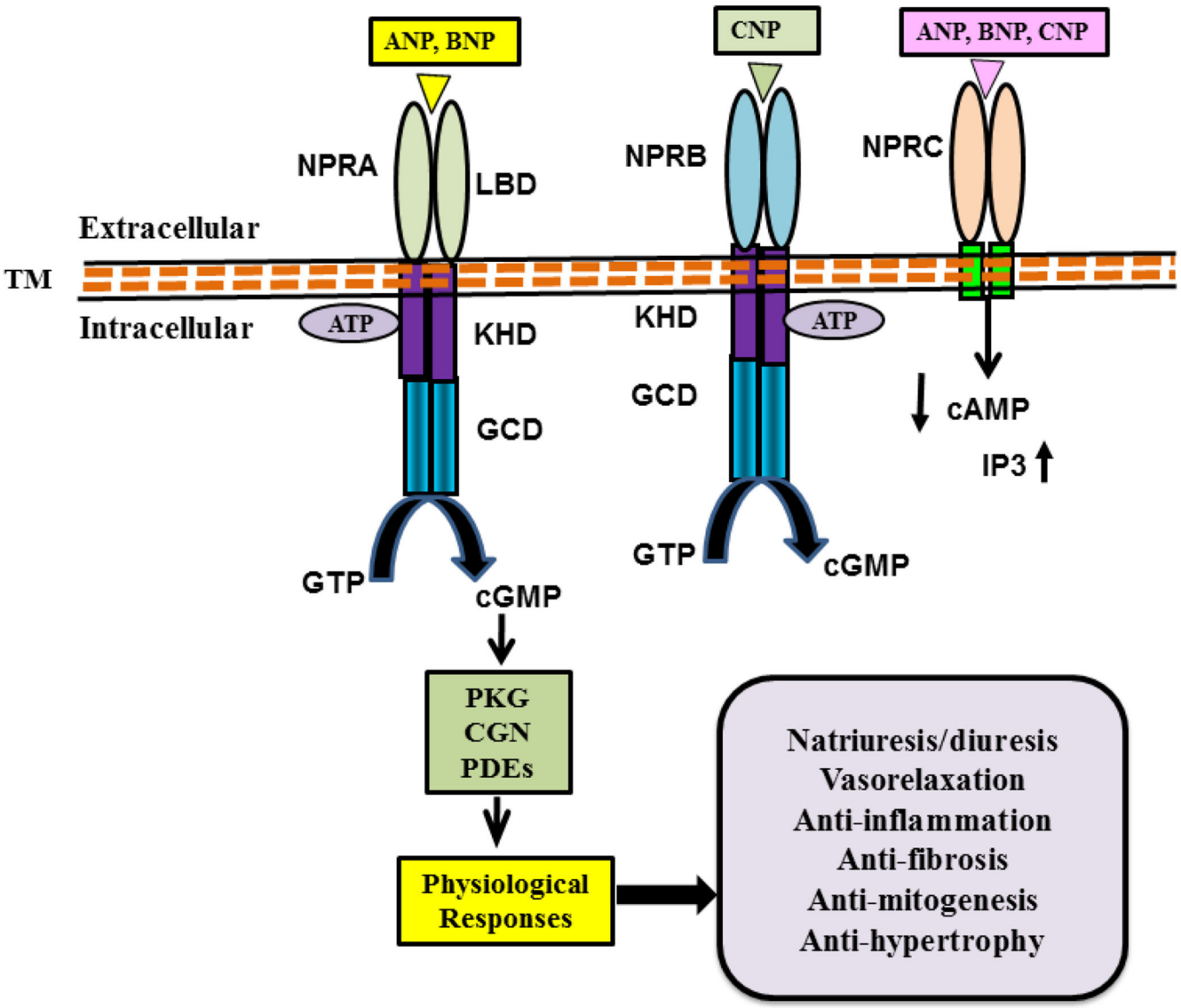

Figure 1. Diagrammatic representation of ligand specificity, transmembrane assembly, and the intracellular signaling system of natriuretic peptide receptors, namely NPRA, NPRB, and NPRC:

The extracellular and intracellular domains of these receptors are shown in different colors. Extracellular LBD and intracellular protein-KHD and GCD domains of NPRA and NPRB are indicated with receptor topology. The extracellular domain and short intracellular tail of NPRC are differentially shown. NPRA and NPRB are represented to bind ATP in the intracellular KHD region of the receptors and to generate second-messenger cGMP from the hydrolytic conversion of GTP to cGMP. The increased level of cGMP activates three known effector molecules: cGMP-dependent PKG, cGMP-gated CNGs, and cGMP-activated PDEs. The activation of PKGs, CNGs, and PDEs elicits natriuretic peptide-dependent physiological responses, including natriuresis/diuresis, vasorelaxation, anti-inflammation, antifibrosis, anti-mitogenesis, and anti-hypertrophy. ANP/BNP binding to NPRC has been shown to decrease cAMP and to increase IP3 in target cells. ANP, atrial natriuretic peptide; ATP,

adenosine triphosphate; BNP, brain natriuretic peptide; CNG, cyclic nucleotide-gated ion channel; CNP, C-type natriuretic peptide; GCB, guanylyl cyclase catalytic domain; GTP, guanosine trisphosphate; IP3, inositol trisphosphate; KHD, kinase homology domain; LBD, ligand-binding domain; NP, natriuretic peptide; PDE, phosphodiesterase; PKG, protein kinase G; TM, transmembrane domain; NPRA, natriuretic peptide receptor-A; NPRB, natriuretic peptide receptor B; NPRC, natriuretic peptide receptor C.

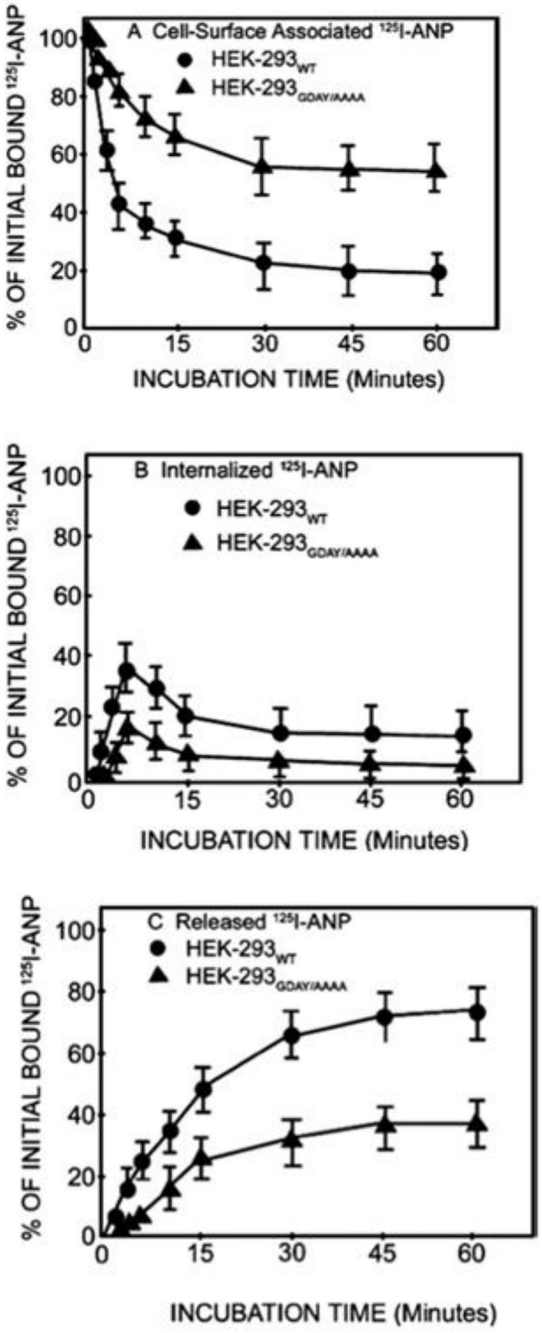

Figure 2. Quantitative analyses of cell-surface-associated, internalized, and released ^{125}I-ANP radioactivity in recombinant HEK-293 cells expressing wild-type or GDAY/AAAA mutant receptors:

Confluent HEK-293 cells expressing wild-type or mutant receptors were allowed to bind ^{125}I-ANP at 4 ^{0}C for 1 h. Cells were washed four times with 2 ml of assay medium to remove unbound ANP, then reincubated in fresh medium at 37 °C. At the indicated time points, cells were placed on ice at 4 ^{0}C and media were collected. Cell-surface-associated (acid-sensitive) radioactivity was eluted with glycine acidic buffer (pH 3.8) and cells were dissolved in 1 M NaOH to determine the internalized (acid-resistant) radioactivity. Cell-

surface-associated (A), internalized (B), and released (C) ^{125}I-ANP radioactivity was determined in the acid eluate, cell extract, and culture medium. Figure adapted from Pandey *et. al.,* 2005 with permission from Biochemical Journal.

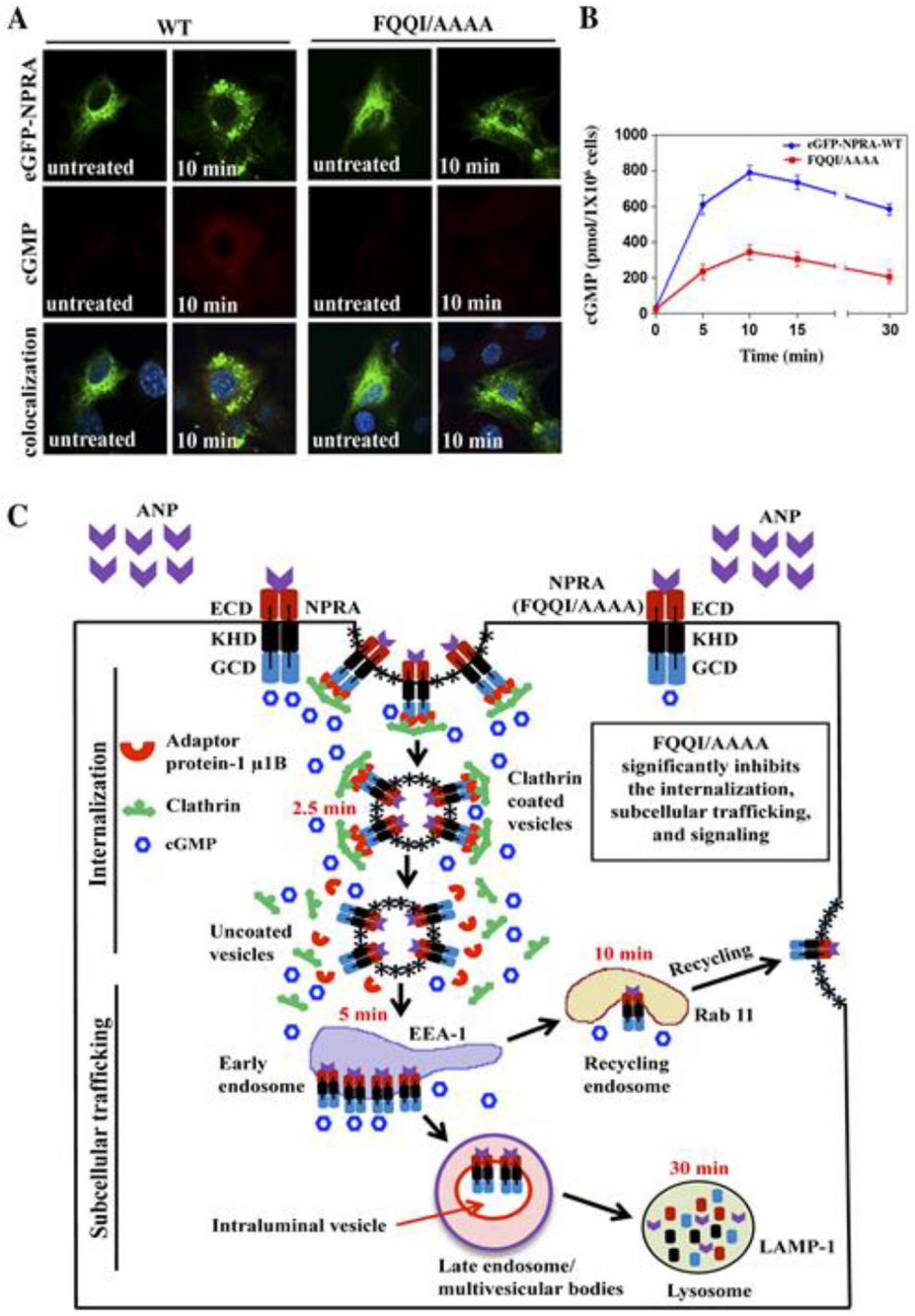

Figure 3. Immunofluorescence localization of cGMP and schematic representation of internalization, intracellular trafficking, and signaling pathway of NPRA in primary MMCs: Immunofluorescence localization of cGMP was done at 48 h after transfection of cells with eGFP-NPRA-WT and mutated motif FQQI/AAAA. To inhibit the endogenous expression of NPRA, cells had been transiently transfected with pcDNA-6.2- GW/GFP-miR expression vector containing *Npr1*-miRNA insert pCMV*Npr1*miRNA-1595 and pCMV-*Npr1*miRNA-2931. The cells were treated with 100 nM ANP for 10 min at 37°C in the presence of IBMX. *A*: After treatment with ANP for 10 min, eGFP-NPRA-WT showed significantly more diffused fluorescence intensity in the cytoplasm than did mutated motif

FQQI/AAAA. *B*: To assay the stimulation of intracellular accumulation of cGMP, cells were transiently transfected with eGFP-NPRA-WT and mutated motif FQQI/AAAA. Intracellular accumulation of cGMP through eGFP-NPRA-WT and mutated motif FQQI/AAAA was quantitated. cGMP assay was performed to determine the concurrent generation of cGMP during receptor internalization and trafficking. Cells were treated with 100 nM ANP for 5, 10, 15 or 30 min in the presence of 0.2 mM IBMX. Cells were washed three times with PBS and scraped in 0.1 M HCl. Cell suspensions were subjected to five cycles of freeze and thaw and then centrifuged at 10 000 x *g* for 15 min at 4 ○ C. The supernatant was collected for cGMP assay using direct cGMP complete ELISA kit (Arbor Assay, Ann Arbor, MI) according to the manufacturer's protocol. *C*: Scheme depicting the sequential events of internalization, trafficking, recycling, and degradation of ligand-receptor complexes in intracellular compartments. After ANP-binding to NPRA, ligand-receptor complexes entered cells via clathrin-coated pits. Ligand-bound receptor complex trafficked intracellularly through endosomes, lysosomes, and a population of receptor recycled back to the plasma membrane through the recycling endosomes, with concurrent generation of intracellular cGMP. Sorting of bound ANP-NPRA complex occurs by endosomal dissociation metabolic and lysosomal degradative pathways. On the other hand, mutation of FQQI residues to AAAA (FQQI/AAAA) significantly inhibited receptor internalization and intracellular trafficking. Note that the multivesicular body formation likely places the receptor in the lumen of the lysosomes. IBMX, 3-isobutyl-1-methylxanthine; ECD, extracellular domain; KHD, kinase homology domain; GCD, guanylyl cyclase domain. Figure adapted from Mani *et. al.,* 2016 with permission from American Journal of Physiology-Renal Physiol.

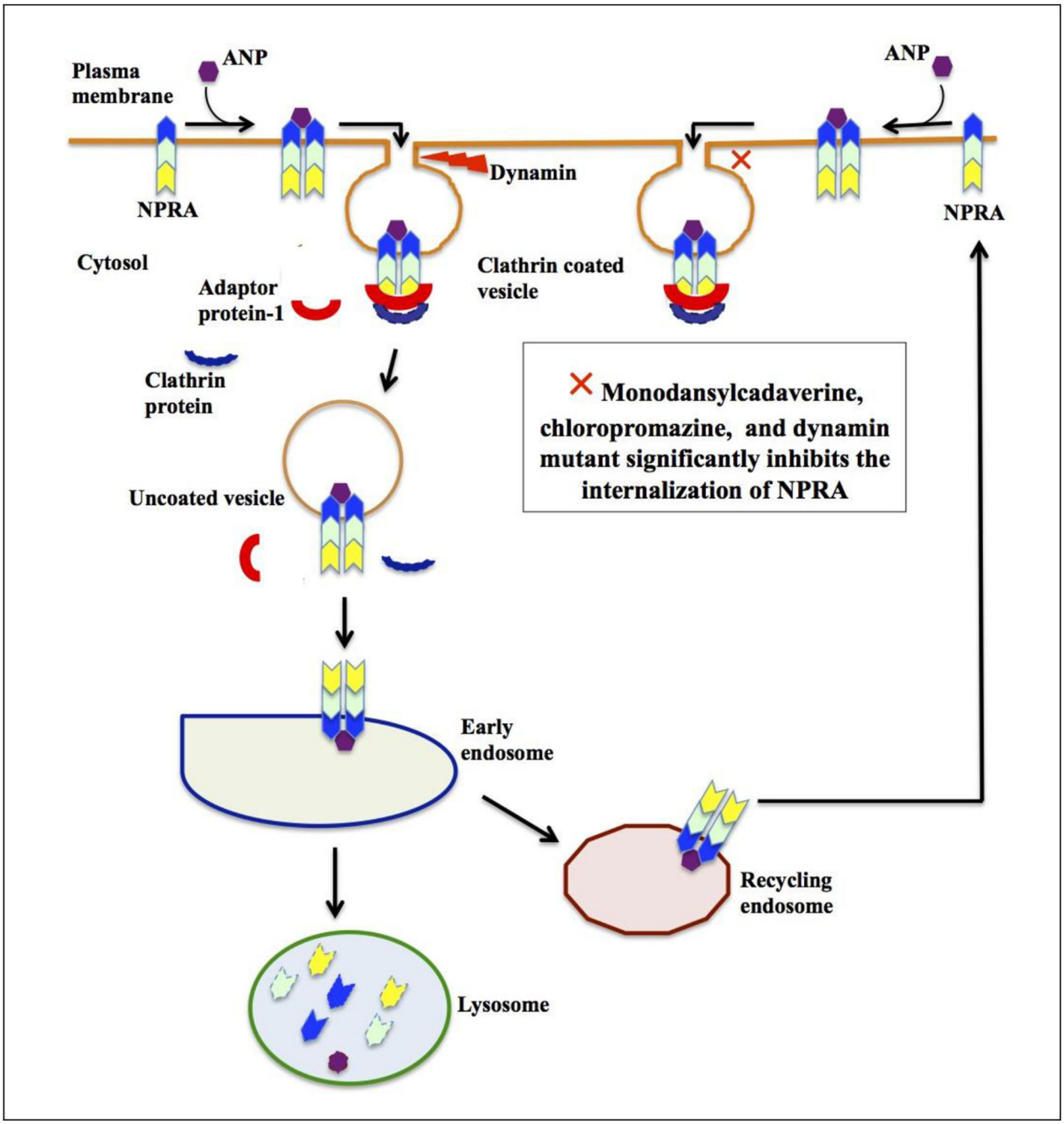

Figure 4. Schematic representation of clathrin-dependent endocytosis of NPRA in recombinant HEK-293 cells:

This scheme describes the sequential events of internalization, trafficking, recycling, and degradation of ligand-receptor complexes in intracellular compartments. After binding of ANP to NPRA, ligand–receptor complexes enter the cell via clathrin-coated pits with the help of dynamin GTPase. The ligand-bound receptor complex is endocytosed and traffics intracellularly through the endosomes and lysosomes. A population of receptor recycles back to the plasma membrane through the recycling endosomes. On the other hand, with the expression of mutant dynamin and the blockers of dynamin GTPase (monodansylcadaverine,

chlorpromazine) inhibiting clathrin-coated vesicle formation, the internalization of NPRA was significantly restricted. Figure adapted from Somanna *et. al.,* 2018 with permission from Molecular and Cellular Biochemistry.

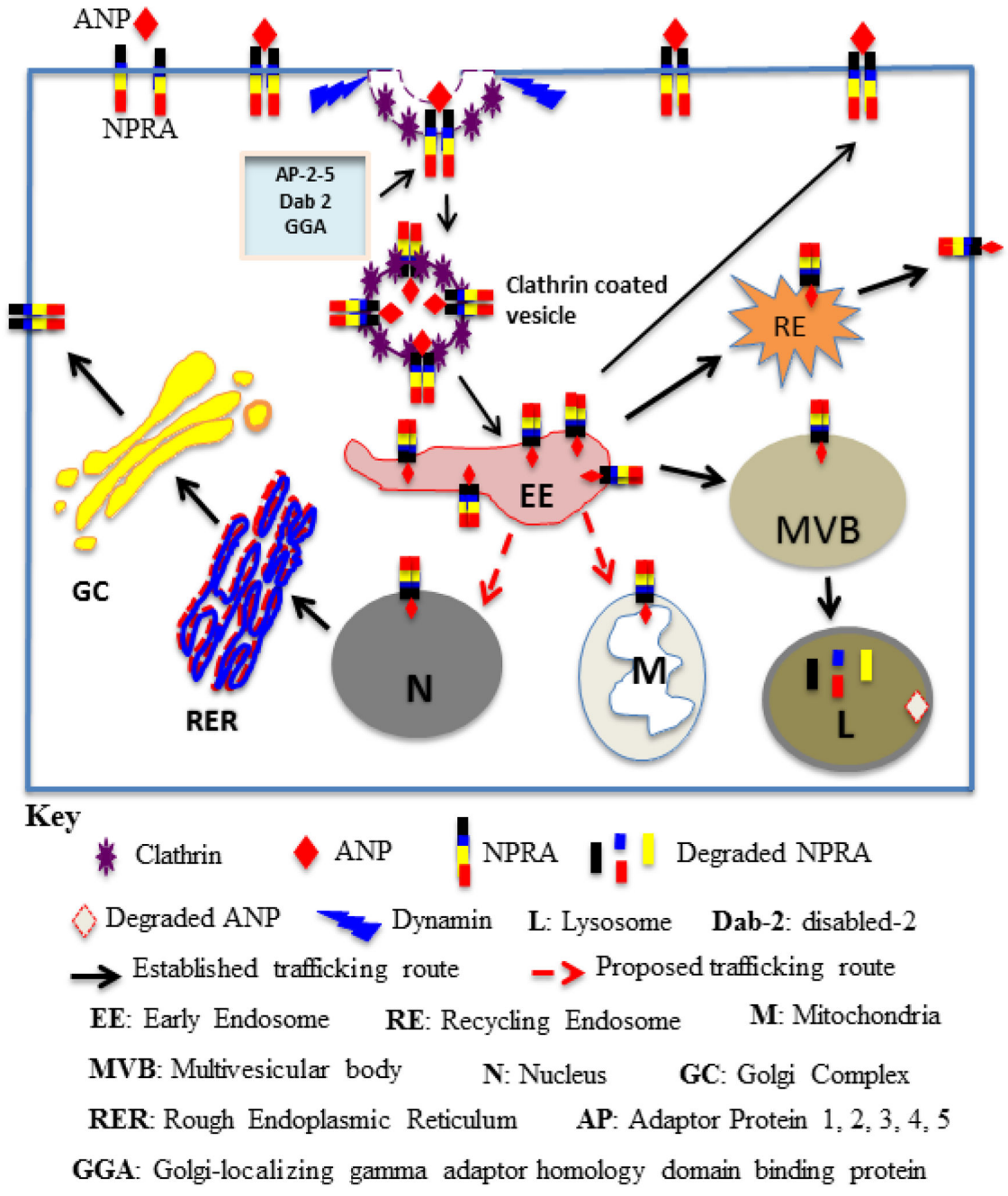

Figure 5. Schematic representation of the proposed intracellular trafficking pathways of NPRA through the multi-organelle systems in cell compartments:

Activated cell-surface NPRA is internalized and sorted at the early endosome. The fate of the receptor, with the recycling endosome, has important consequences for biological responses, although the degradative pathway is via multivesicular bodies (MVBs)/lysosome. Typical pathways to the nucleus and mitochondria have been proposed to favor survival, but the transport mechanisms have not yet been established. Conversely, ligand-activated ANP-NPRA complexes recycle back on plasma membranes for re-endocytosis.

Table 1.

A list of important short-sequence signal motifs for the internalization and trafficking of membrane receptors and proteins.

Membrane Receptor/Protein	Signal Motifs	Targeted Pathways	Reference
Membrane Receptors:			
CD-Mannose-6-phosphate receptor	YKYSKV	Endocytosis/lysosomal targeting	[185]
CI-Mannose-6-phosphate receptor	YSKV	Endocytosis/lysosomal targeting	[136]
Glutamate receptor	YWL	Endocytosis	[146]
GC-A/NPRA	GDAY	Endocytosis	[62]
GC-ANPRA	FQQI	Endocytosis	[26]
Insulin-like growth factor receptor	YxxPhi	Endocytosis	[136, 185]
LDL receptor	FDNPVY	Endocytosis	[145]
LH receptor	GTALL	Endocytosis	[186]
LDL-related receptor	YATL	Endocytosis	[187]
Mannose phosphate receptor	FENTLY	Lysosomal targeting	[136, 185]
Platelet activating factor receptor	[YF]xNPx[YF]	Endocytosis	[188]
Protease-activated receptor-1	YKKL	Endocytosis	[140]
P2X receptor	YEQGL	Endocytosis	[189]
Transferrin receptor	YTRF/Q	Endocytosis	[136, 185]
T-cell receptor	YQPL	Endocytosis	[136]
Membrane Proteins:			
Acetylcholine transporter	DSLL	Endosomal targeting	[147]
Beta-amyloid precursor protein	YENPTY	Endocytosis	[190]
CD3 Chains	[DE]xxxL[LI]	Endocytosis	[191]
Integrin	NPxY	Endocytosis	[192]
GLUT4	FQQI	Endocytosis/lysosomal targeting	[193]

The evidence suggest that various short sequence signal motifs usually contain tyrosine or phenylalanine residues, followed by hydrophobic and/or aromatic residues. Certain sequence motifs also contain acidic residues with required amino acid residue tyrosine. Membrane receptors and proteins also make the use of dileucine short signal motifs. GC-A, guanylyl cyclase-A; NPRA, natriuretic peptide receptor-A; LDL, low density lipoprotein; LH, leutinizing hormone; x, refers to any amino acid residue; P2X receptor, purinergic P2Xreceptor; Phi, refers to hydrophobic amino acid residue; GLUT4, glucose transporter 4.