Neuregulin Signaling in the Heart
Dynamic Targeting of erbB4 to Caveolar Microdomains in Cardiac Myocytes

You-Yang Zhao, Olivier Feron, Chantal Dessy, Xinqiang Han, Mark A. Marchionni, Ralph A. Kelly

Abstract—Two of the neuregulins (NRG1 and NRG2) and their receptors (erbB2 and erbB4) are essential for normal cardiac development and can mediate hypertrophic growth and enhance survival of embryonic, postnatal, and adult rat ventricular myocytes. The expression of erbB4, the predominant NRG receptor in postnatal rat ventricular muscle, declines after midembryogenesis, and its expression is limited to cardiac myocytes. A full-length erbB4 rat cDNA isolated from neonatal ventricular muscle was found to be highly homologous to human erbB4 and contained a caveolin binding motif within the cytoplasmic kinase domain. Using the complementary techniques of detergent-free density-gradient ultracentrifugation of myocyte lysates and coimmunoprecipitation of erbB4 and caveolin-3, the caveolin isoform expressed in cardiac myocytes, erbB4 could be localized (using both approaches) to caveolar microdomains. Moreover, addition of a soluble NRG1, recombinant human glial growth factor 2, resulted in rapid (2-minute) translocation of erbB4 out of caveolar microdomain in cardiac myocytes. Thus, erbB4 is dynamically targeted to caveolar microdomains within cardiac myocytes. Its rapid translocation after NRG1 binding may contribute to receptor desensitization in the continuous presence of ligand. (Circ Res. 1999;84:1380-1387.)

Key Words: caveolin ■ cardiac myocyte ■ signal transduction ■ neuregulin

Neuregulin (NRG)–erbB signaling has been shown to play an essential role in inducing trabeculation and valvuloseptal formation in the developing myocardium.1–5 The erbB receptor family, in addition to erbB1/epidermal growth factor receptor (EGFR), is composed of 2 direct receptors, erbB3 and erbB4, and 1 coreceptor, erbB2/neu.6 Members of the NRG1 family of signaling proteins, such as glial growth factors, heregulins, and neu differentiation factor, as well as NRG2, are agonists for erbB3 and erbB4, whereas NRG3 appears to be selective for erbB4. In addition to ligand specificity and affinity and their expression patterns, the 4 erbB receptors differ in their ability to transduce ligand-activated signaling (eg, erbB3 has limited kinase activity).7 Ligands that specifically bind to the erbB1, including EGF and several EGF-like gene products, do not directly interact with erbB2 or erbB3, although several recent reports have documented binding of heparin-binding EGF, betacellulin, and epiregulin to erbB4.8,9 All NRG1 isoforms bind with low affinity to erbB3 and with high affinity to erbB4.10 Although no specific ligand for erbB2 has been identified, heterodimers of erbB2 and erbB3 have been reported to constitute a second high-affinity binding site for NRGs.7

Ligand-induced desensitization and downregulation mechanisms are important aspects of the regulation of transmembrane receptors.11 Ligand binding to the erbB1 receptor rapidly induces receptor-mediated endocytosis through clathrin-coated pits,12 and the internalized complexes are subsequently degraded in lysosomes. In contrast to the erbB1/EGFR, all other erbB family members, including erbB4, are not rapidly internalized in the presence of ligand,13 although Vecchi and Carpenter14 have demonstrated recently that activation of a protein kinase C isoenzyme in a number of cell types that constitutively express erbB4, including the AT1 cardiac muscle–like cell line, results in proteolytic cleavage of the 120-kDa ectodomain of the receptor, probably by a metalloproteinase. The membrane-spanning and cytoplasmic domains were subsequently shown to undergo ubiquination and targeting to the proteasome.14

Recently, the erbB1/EGFR was identified to directly interact with caveolins in caveolae in mammalian A431 cells.15 Caveolae are small flask-shaped invaginations of the plasma membrane, characterized by high levels of cholesterol and glycosphingolipids that mediate transcytosis and endocytosis by clathrin-independent sequestration pathways,16,17 and are found in most cell types, including smooth, skeletal, and cardiac muscle.18,19 Caveolar localization of a number of signaling pathways has been identified, which, among other functions, appears to facilitate ligand-mediated activation of

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From the Cardiovascular Division (Y.-Y.Z., X.H., R.A.K.), Brigham and Women’s Hospital and Harvard Medical School, Boston, Mass; Department of Medicine (O.F., C.D.), University of Louvain, Brussels, Belgium; and Cambridge Neuroscience, Inc (M.A.M.), Cambridge, Mass. The current affiliation for Y.-Y.Z. is the Department of Medicine, University of California at San Diego, School of Medicine, La Jolla, Calif. Correspondence to Ralph A. Kelly, Cardiovascular Division, Brigham and Women’s Hospital, 75 Francis St, Boston, MA 02115. E-mail rakelly@rics.bwh.harvard.edu
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signal transduction and cross talk between distinct signaling cascades.17–19

The principal scaffolding proteins of caveolae are the caveolins, 20- to 24-kDa integral membrane proteins that form oligomultimers.20 The following 3 caveolin isoforms have been identified to date: caveolin-1 (α and β), caveolin-2, and caveolin-3. Caveolin-1 is found in a variety of cell types, whereas caveolin-2 is expressed primarily in adipocytes.21 Caveolin-3 is expressed predominantly in striated muscle and is the only isoform that has been identified in cardiac myocytes.22,23 All caveolins share common cytoplasmic scaffolding domains that mediate the interactions of caveolins with themselves and other proteins.24–26 Furthermore, 2 caveolin-binding motifs, $\phi x\phi x\phi x\phi x\phi$ and $\phi x\phi x\phi x\phi x\phi$ ( $\phi$ represent the aromatic amino acids Trp, Phe, or Tyr), have been identified and found in most caveolin-associated proteins.27 The motif WSYGVTIW within the kinase domain of erbB4 has been identified and found in most caveolin-associated proteins.27 The motif WSYGVTIW within the kinase domain of erbB4 (corresponding to the codon positions 896 to 1262) was labeled with [3H]dCTP by random priming and used as a probe in Northern blots. A rat cardiac myosin light chain 2 (MLC-2v) cDNA probe or rat SS rRNA oligonucleotide (5'-ACGGTATCTGAT-3') was used as control.

To determine the protein level of erbB4 during development, hearts from embryonic (E14), postnatal day 1 (P1), and adult rats were homogenized in the lysis buffer.28 One hundred micrograms of each lysate was fractionated by SDS-PAGE, transferred onto a PVDF membrane, and probed with an erbB4-specific antibody. To detect erbB4 protein in adult rat hearts, 2000 μg of the lysates were first immunoprecipitated with an erbB4-specific antibody and then probed with the same anti-erbB4 antibody. Signal intensities were determined by densitometry (Ultrascan XL, Amersham Pharmacia Biotech).

Subfractionation of Cardiac Myocyte Lysates

NRVMs maintained in serum-free medium for 2 days were treated either without or with rhGGF2 (30 ng/mL) and then scraped in a freshly prepared solution of 200 mM NaCl, and lysed by sonication (three 5-second bursts, minimal output power) using a Branson sonifier 450 (Branson Ultrasonic Corp) according to a method modified from Song et al.30 The cell lysates were then adjusted to 35% sucrose by addition of a sucrose stock solution prepared in MBS (25 mMol/L MES, pH 6.5, and 150 mMol/L NaCl) and loaded at the bottom of a 5%–10%–20%–30% discontinuous sucrose gradient (in MBS containing 100 mMol/L NaCl, NaCO₃) for an ultracentrifugation (150 000g). The gradient was fractionated into 10 fractions that were subsequently neutralized with HCl before analysis. Mannosidase II activity and [3H]ouabain binding as markers for Golgi and sarcoulemmal membranes, respectively, were assayed as previously described.31

Analysis of erbB4 Expression in Rat Heart

Total cellular RNA was isolated by a modification of the acid guanidinium/thiocyanate phenol/chloroform extraction method using the Trizol reagent (Life Technologies, Inc). The 3'-fragment of rat heart erbB4 (corresponding to the codon positions 896 to 1262) was labeled with [3H]dCTP by random priming and used as a probe in Northern blots. A rat cardiac myosin light chain 2 (MLC-2v) cDNA probe or rat SS rRNA oligonucleotide (5'-ACGGTATCTGAT-CGTTCTCGAACC-3') was used as control.

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Materials and Methods

Preparation of Cardiac Myocyte and Nonmyocyte Primary Cultures

Neonatal or adult rat ventricular myocyte (NRVM or ARVM) primary cultures and coronary microvascular endothelial cells (CMECs) from adult rat hearts were prepared as described previously.28 Primary cultures of cellular fractions isolated from neonatal hearts enriched in nonmyocyte cells were prepared by passing twice cells that adhered to the tissue culture dish during the preplating procedure. These nonmyocyte cultures, which contained few anti-myosin heavy chain–positive cells, were allowed to grow to subconfluence in DMEM supplemented with 20% FBS before switching to DMEM plus ITS for a subsequent 36 to 48 hours.

Molecular Cloning of Rat Heart erbB4

A cDNA library was constructed in a ZAP II (Stratagene, La Jolla, CA) from oligo(dT)-primed rat heart RNA. erbB4-specific clones were isolated by probing the library with the [3H]dCTP-labeled 3'-fragment of ErbB4 amplified from NRVMs with primers erbB4A and erbB4B.28 All cDNA clones were sequenced on both strands by automatic DNA sequencing.

Overexpression and Activation of Rat Heart erbB4 in COS-7 Cells

A full-length erbB4 expression vector under control of the cytomegalovirus (CMV) immediate-early promoter was generated by cloning the erbB4 open reading frame between the unique sites Nhel and Smal of pBK-CMV (Stratagene). This expression vector and pBK-CMV vector were transfected into COS7 cells by lipofectin reagents (Life Technologies, Inc). All cells were routinely grown in DMEM. At 48 to 72 hours after transfection, transfected cells (80% confluent) in 6-well culture dishes were starved overnight in 0.2% BSA and then treated with 30 ng/mL of recombinant human glial growth factor 2 (rhGFF2) (Cambridge Neuroscience Inc) for 10 minutes. Cells were quickly rinsed twice with ice-cold PBS and lysed in cold lysis buffer.28 Lysates were centrifuged at 12 000g at 4°C for 20 minutes, and 300-μg aliquots of cellular protein were immunoprecipitated by an erbB4-specific antibody (Santa Cruz Biotechnology, Inc) overnight at 4°C. Immunoprecipitates were collected, fractionated, transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), and probed with a PY99 anti-phosphotyrosine antibody or ErbB4-specific antibody (Santa Cruz Biotechnology, Inc).

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analysis with an anti–caveolin-3 monoclonal antibody (mAb) probe (Transduction Laboratories) or an erbB4-specific antibody (Santa Cruz Biotechnology, Inc).

Results

Cloning and Expression of erbB4 in Cardiac Muscle

We previously reported that erbB4 was the most prevalent receptor for NRGs expressed in the postnatal myocardium. A full-length cDNA of erbB4 was isolated by screening a rat heart bacteriophage cDNA library with a rat heart erbB4 cDNA probe. Nucleotide sequence comparison revealed a 90% (3666/4060) identity with the human erbB4. The deduced amino acid sequence of rat erbB4 and the sequence deviations between human and rat erbB4 are shown in Figure 1. Overall, there was 96% amino acid identity between the human and rat sequences. The 276-amino acid tyrosine kinase domain is the most conserved region, whereas the extreme C terminus is more variable, although all the C-terminal tyrosines, including the major tyrosine autophosphorylation sites, are conserved. The rat erbB4 gene product is predicted to consist of 1308 amino acids. When overexpressed in a mammalian cell line (COS-7 cells), the erbB4 could be tyrosine phosphorylated by a soluble NRG1 (rhGGF2) (Figure 2).

Using an erbB4-specific cDNA probe (encoding codon positions 896 to 1262), erbB4 was found to be expressed in both atrial and ventricular muscle by Northern blot (Figure 3A). The mRNA level of erbB4 in the atrium was ∼3-fold higher than that in ventricle muscle. As shown in Figure 3B, erbB4 was identified only in neonatal and adult ventricular myocytes, not in primary cultures of CMECs or in a myocyte-depleted cell population (ie, nonmyocytes from postnatal day 1 rat heart), which, using the method of myocyte isolation we used here, is composed largely of fibroblasts and endothelial cells.

To investigate the expression profile of erbB4 receptor during heart development, total RNAs were extracted from the whole hearts from midembryogenic, neonatal, and adult rats. As shown in Figure 4A, the mRNA level of erbB4 was relatively high in embryonic (E14) myocardium, but declined by half in neonatal rat heart (P1) and by 90% in adult myocardium.
ventricular muscle. Using an erbB4-specific antibody, measurement of erbB4 protein revealed a similar pattern. As shown in Figure 4B, the erbB4 protein was much more readily detectable in embryonic (E14) myocardium than in adult myocardium. There was a ~4-fold greater erbB4 protein abundance in E14 myocardium than that in postnatal (P1) myocardium.

Cofractionation of erbB4 With Caveolin-3 in Cardiac Myocytes
To examine the potential association of erbB4 receptor with caveolin-3, caveolin-enriched membranes were isolated by a detergent-free purification method based on the resistance to extraction of caveolin complexes by sodium carbonate and on the fine disruption of cellular membranes by sonication.31 Thus, after homogenization of NRVMs in a sodium carbonate buffer, myocyte lysates were adjusted to a 35% sucrose content and placed at the bottom of a 5%–10%–25%–30% sucrose density gradient for an overnight ultracentrifugation. Aliquots of the fractions collected were separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with anti–caveolin-3 or anti-erbB4 antibodies. As displayed in Figure 5A, the majority (~80%) of caveolin-3 and erbB4 in neonatal ventricular myocytes appeared in fractions 2, 3, 4, and 5, which corresponded to the 5% to 15% sucrose equilibrium densities. The gradient fractions were also analyzed for their protein content as well as for the presence of mannosidase II, as a Golgi marker, and for the level of specific [3 H]ouabain binding, as a marker for sarcolemmal NaK-ATPase.31 As shown by the pattern of distribution of these markers across the gradient (Figure 5B), the...
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bulk of cellular proteins equilibrate at the high sucrose density (fraction 10), corresponding to Golgi and sarcosomal membranes. The small amount of caveolin-3 and erbB4 associated with these high-density fractions (Figure 5A) is possibly due to some association of both proteins with the trans-Golgi network or to incomplete cell lysis before sucrose density gradient centrifugation.

We also performed a coimmunoprecipitation and immunoblotting experiment to investigate interactions between erbB4 and caveolin-3 in cardiac myocytes. Using protocols we have previously used to explore eNOS targeting to caveolae,

Figure 5. Association of erbB4 receptor with caveolin in NRVMs. A and B, Association of erbB4 receptor with caveolin-enriched microdomains in NRVMs. After isopycnic centrifugation of NRVMs on sucrose gradients as described in the text, aliquots of 1-mL fractions were resolved by SDS-PAGE (12.5% acrylamide), transferred onto PVDF membranes, and immunoblotted with either an anti-caveolin-3 mAb or an anti-erbB4 pAb. Fraction 1 refers to the top of the gradient. These data represent the result of a typical fractionation experiment (A). B, The distribution of total protein (●), plasma membrane (▲), and Golgi (●) markers along the sucrose density gradient is shown. Mannosidase II activity and [3H]ouabain binding have been used as specific markers of the Golgi and sarcosomal membranes, respectively. The data represent the results of a typical fractionation procedure with individual measurements performed in triplicate. C, Coimmunoprecipitation of erbB4 with caveolin-3 in NRVMs. Cell lysates were prepared from confluent NRVMs in primary cultures after 2 days in serum-free medium and were immunoprecipitated with either an anti-erbB4 antibody or an anti-caveolin-3 antibody (C3, positive control), or a nonimmune IgG1 control antibody (IgG, negative control). Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblot analysis with a mAb directed against caveolin-3.

Ligand-Induced Translocation of erbB4 Receptor From Caveolae

To determine whether a change in erbB4 receptor subcellular localization could be induced by agonist binding, we examined the effects of rhGGF2 on the distribution of erbB4 receptors using the centrifugation protocol described above. Subconfluent NRVMs, maintained in serum-free medium for 2 days, were treated with 30 μg/mL (ie, 0.54 nmol/L) of GGF2 for 5 minutes. After extensive washing, myocytes were lysed and submitted to isopycnic centrifugation on a sucrose gradient. Unlike the data reviewed above from untreated cardiac myocytes (Figure 5A), <10% of erbB4 could be detected in caveolin-3–enriched fractions (fractions 2, 3, and 4), whereas the majority of erbB4 could be detected only in the high-density fractions (fractions 8, 9, and 10) in which relatively little caveolin-3 could be fractionated (Figure 6A). These experiments suggest that erbB4 is translocated from caveolae on agonist binding. In contrast to erbB4, the erbB2 receptor remained in caveolae after agonist binding (Figure 6A).

To confirm the dynamic targeting of erbB4 receptor to caveolae in cardiac myocytes, a series of coimmunoprecipitation and immunoblotting experiments was performed to investigate interactions between caveolin-3 and erbB4 in cardiac myocytes. We used an erbB4-specific antibody for immunoprecipitation and then identified caveolin-3 using a caveolin-3–specific antibody. Cardiac myocytes, after 2 days in serum-free medium, were incubated with or without rhGGF2 (30 ng/mL) for the indicated time points and then lysed and resolved on PVDF membranes. Figure 6C shows that the level of erbB4-immunoprecipitated caveolin-3 in rhGGF2-treated cardiac myocytes had decreased to ≈20% of the level in untreated cells at 2 minutes. Alternatively, the cell lysates were immunoprecipitated with a caveolin-3–specific pAb and then immunoblotted with an erbB4–specific antibody. As shown in Figure 6D, the level of caveolin-3–associated erbB4 protein in cardiac myocytes quickly decreased after stimulation with rhGGF2 (30 ng/mL). Within 5 minutes, caveolin-3–associated erbB4 protein was difficult to detect. These results confirm that erbB4 receptor rapidly dissociates from caveolin on transactivation by NRGs in cardiac myocytes.
To explore the proportion of erbB4 associated with caveolin-3 in cardiac myocytes, we analyzed erbB4 immunoblots of the supernatants remaining after pelleting the protein A-bound immunocomplexes that had been previously precipitated from the solubilized cell lysates by the caveolin-3-specific antibody (as analyzed in Figure 6D). As shown in Figure 6E, in the lysates of untreated cardiac myocytes, little erbB4 protein remained in the supernatant after immunoprecipitation with the caveolin-3-specific antibody, indicating that all or the majority of erbB4 is associated with caveolin-3 in these cells. In contrast, erbB4 protein quickly dissociated from caveolin-3 in cardiac myocytes treated with rhGGF2. The lost caveolin-3-associated erbB4 protein in Figure 6D could quantitatively be recovered from the supernatants (Figure 6E).

Discussion

erbB4 was initially isolated from a human breast cancer cell line MDA-MB-453, and exhibits the structural motifs common to many receptor tyrosine kinases. Compared with the human homologue, rat heart erbB4 has a highly conserved tyrosine kinase domain and all of the C-terminal tyrosines, including the major autophosphorylation sites (Figure 1). By in situ hybridization, erbB4 transcripts were originally determined to be confined to cardiac muscle and the nervous system in E9.5 mouse embryos. Within the heart, erbB4 mRNA was noted to be present throughout both the atrial and ventricular myocardia, but it is absent from the developing endocardium. As we have documented here, erbB4 was found to be expressed in both atrial and ventricular myocytes in adult rat heart, with a ≈3-fold greater abundance in atrial muscle than in the ventricle. Different NRG isoforms also have been identified recently in atrial and ventricular muscle. NRG1-derived mRNAs are limited largely to the endocardial endothelium of developing ventricular muscle, whereas NRG2 mRNAs are found primarily in the endothelium of developing atrium. Moreover, as we have shown here, erbB4 appears to be expressed specifically in cardiac myocytes and not in myocyte-depleted “nonmyocyte” cell populations, including the microvascular endothelium, in both neonatal and adult rat myocardium.

We have used 2 complementary approaches to investigate the subcellular localization of erbB4 receptor in cardiac myocytes. In the absence of ligand binding, this receptor tyrosine kinase was localized to caveolae as determined both by density gradient centrifugation of myocyte lysates and by coimmunoprecipitation of caveolin-3 with erbB4. In the presence of NRG1, erbB4 rapidly translocated out of caveolae.

Figure 6. NRG1 induces dissociation of erbB4 from caveolin-3 in NRVMs. A and B, NRG1 induces translocation of erbB4, but not erbB2, out of the caveolar microdomains in NRVMs. Fractionation experiments similar to those described in Figure 5A and 5B were done on NRVMs pretreated with rhGGF2 for 5 minutes. These experiments were repeated twice, with similar results. Note that the membrane pellet obtained after ultracentrifugation became detached from the bottom of the tube and was collected in fraction 9 instead of 10 (Figure 5A). C through E, NRG1 induces dissociation of erbB4 protein from caveolin-3 in NRVMs. NRVMs maintained in a serum-free medium for 2 days were treated either without (C, control) or with the soluble NRG1, rhGGF2 (30 ng/mL), for the indicated times and then collected and lysed. An aliquot of each cell lysate was immunoprecipitated with an anti-erbB4 antibody and probed with an anti–caveolin-3 mAb. The same blot was stripped and reprobed with the anti-erbB4 antibody (C). A complementary experiment was done with a similar procedure. The cell lysates were immunoprecipitated with a caveolin-3–specific pAb and probed with anti-erbB4 antibody. The same blot was stripped and reprobed with an anti–caveolin-3 mAb (D). After immunoprecipitation with the anti–caveolin-3 antibody, the remaining supernatant was reimmunoprecipitated with an erbB4–specific antibody and then probed with the same erbB4 antibody (E). The signal density was scanned by densitometry. The experiment shown is representative of 3 independent experiments.
lae. Interestingly, we found that erbB2, another NRG receptor expressed in the postnatal rat heart, also cofractionated with caveolea in NRVMs. In contrast to erbB4, erbB2 was found not to translocate out of caveolea after NRG1 stimulation (Figures 5 and 6). To date, a number of receptor tyrosine kinases, as well as their downstream signaling targets, have also been localized to caveolea or caveola-related domains,34–38 which suggests that there could be a structural motif common among receptor tyrosine kinases that mediates their interaction with the caveolins. Indeed, a highly conserved motif, DVWSYGVTVWELMT (residues 894 to 907 in erbB1/EGFR), has been identified to be responsible for caveolin binding.14 However, unlike the EGFR, which remained in caveolea after stimulation of the A431 cells with EGF,14 the erbB4 receptor in cardiac myocytes rapidly dissociated from caveolea-3 and the caveolar microdomain in response to treatment with rhGGF2. Similarly, the platelet-derived growth factor receptor has been localized to caveolea in quiescent normal human fibroblasts and began to translocate out of caveolae after ligand binding, although with a time course somewhat less rapid than described here.34

However, not all receptors translocate out of caveolar microdomains on agonist binding. We have demonstrated previously that the m2 muscarinic cholinergic receptor rapidly translocates into caveolar microdomains after agonist binding, at least in cardiac muscle cells, where, among other effects, it activates the isoform of nitric oxide synthase (NOS) originally described in endothelial cells (ie, eNOS or NOS3).31 The eNOS-caveolin heteromeric complex has been demonstrated to undergo cycles of dissociation and reassociation that are regulated by Ca2+ and calmodulin.39

The rapid dissociation from caveolin-3 and translocation out of caveolar microdomains by erbB4 on NRG binding that we have demonstrated here in cardiac myocytes may facilitate phosphorylation of downstream target proteins. It is also possible that rapid trafficking of the erbB4 ligand complex out of caveolea promotes initial receptor desensitization, with subsequent recycling back into caveolar microdomains after ligand dissociation. These data also indicate that erbB2, which is known to form heterodimers with erbB4 in cardiac myocytes,28 does not translocate out of caveolea on ligand binding, which supports the hypothesis that ligand-induced translocation of erbB4 terminates signaling by the erbB2/erbB4 heterodimer. Further research is needed to validate this hypothesis and clarify the role of ligand-mediated translocation in receptor downregulation.

Acknowledgments

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