Postendocytotic traffic of the galanin R1 receptor: A lysosomal signal motif on the cytoplasmic terminus

Sheng Xia*[†], Xing-Peng Dun*, Ping-Sheng Hu[‡], Svend Kjaer*, Kang Zheng*, Yu Qian*, Christina Solén*, Tao Xu[§], Bertil Fredholm[‡], Tomas Hökfelt*[¶], and Zhi-Qing David Xu*[†‖]

*Departments of Neuroscience and [§]Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden; [†]Beijing Institute for Neuroscience, Beijing Center for Neural Regeneration and Repairing, Capital Medical University, Beijing 100069, China; and [‡]School of Life Science, Huazhong University of Science and Technology, Wuhan 430074, China

The neuropeptide galanin R1 receptor (GalR1) was tagged at its C terminus with EGFP (GalR1–EGFP) to study receptor localization and trafficking. In PC12 and HEK293 cells, functional GalR1–EGFP was expressed on the plasma membrane and internalized into cytoplasmic vesicles after galanin stimulation. The internalization was blocked by 0.4 M sucrose and by silencing of clathrin with siRNA methodology. Internalized GalR1–EGFP and LysoTracker, a lysosomal marker, overlapped in intracellular vesicles after prolonged galanin stimulation. This colocalization was strongly reduced after site-directed mutagenesis of the motif YXXØ on the C terminus of GalR1 (where Ø is a bulky hydrophobic residue and X any amino acid). Taken together, these data suggest that GalR1 is internalized via the clathrin-dependent, endocytic pathway and then, to a large extent, delivered to lysosomes for degradation through the lysosome-targeting signal YXXØ.

degradation | endocytosis | G protein-coupled receptor | green fluorescent protein | internalization

Fig. 1. Galanin-induced internalization of GalR1–EGFP and GalR2–EGFP on transfected PC12 cells. Cells transfected with GalR1–EGFP (*A*) or GalR2–EGFP (*E*) were incubated with galanin (*B* and *F*), AR-M961 (*C* and *G*), or AR-M1896 (*D* and *H*) for 15 min at 37°C. The inset represents a higher magnification of the boxed area, and arrows indicate GalR1–EGFP-containing intracellular vesicles in the perinuclear region. Note that AR-M1896 caused internalization of GalR2–EGFP but not GalR1–EGFP. (Scale bar: 5 μm.)

Galanin, a 29-aa neuropeptide (1), is involved in numerous physiological and pathological neuronal functions, for example, learning and memory (2–4), mood (5–9) and pain (10, 11) control, feeding behavior (12, 13), and neuronal survival and regeneration (14). The galanin R1 receptor (GalR1) is one of three receptor subtypes that have been cloned to date and belong to the seven-transmembrane, G protein-coupled receptor (GPCR) superfamily (15). GalR1 is widely distributed in the central nervous system, including the noradrenergic locus coeruleus neurons (16). Here, GalR1 mediates galanin-induced hyperpolarization associated with a marked desensitization (17–21).

Intracellular trafficking of GPCRs has been extensively studied (22, 23), but galanin receptors to only a limited extent. Thus, internalization of GalR1 receptor was first seen in Chinese hamster ovary (CHO) cells by using fluorescein–*N*-galanin and flow cytometry (24) and subsequently reported in CHO (25) and PC12 (26) cells with GalR1-fluorescent protein constructs. However, to date, very little information has been available on postendocytotic trafficking of GalR1. In the present study, we analyzed mechanism(s) mediating internalization and intracellular trafficking of GalR1, focusing on the molecular factors required for translocation of the agonist-occupied GalR1 from the membrane to cytosolic compartments.

Results

Expression and Internalization of GalR1–EGFP in Transfected Cells.
A chimeric protein was created by fusing EGFP to the C-terminal of the GalR1 (GalR1–EGFP). After stable transfection into a PC12 cell line and analysis with a confocal laser scanning microscope, GalR1–EGFP was predominantly associated with the plasma membrane, but some intracellular vesicles in the perinuclear region also contained the fusion protein (Fig. 1*A*). In contrast, an N-terminal EGFP–GalR1 chimera was not inserted into the plasma membrane (data not shown). After incubation with galanin (100 nM) at 37°C for 10–15 min, GalR1–EGFP was
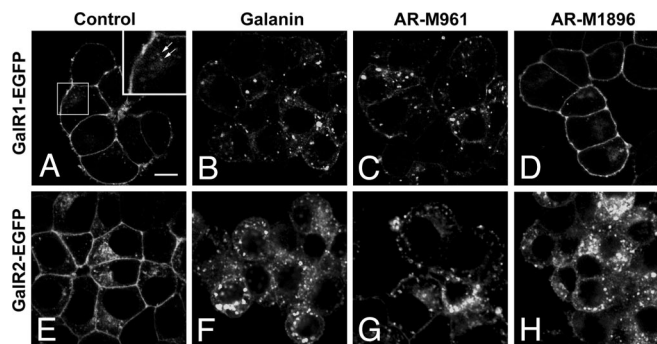
dramatically reduced at the plasma membrane and internalized into numerous vesicles in the cytoplasm (Fig. 1*B*), distinctly seen at a concentration of 10^{-8} M (Fig. 2), demonstrating a dose-dependent, ligand-induced endocytosis of the chimeric protein. Similar results were obtained for GalR1–EGFP in HEK293 cells (data not shown). For comparison, PC12 cells stably transfected with a GalR2–EGFP construct (27) were studied, also showing membrane localization (Fig. 1*E*) and galanin-induced internalization of the receptor (Fig. 1*F*).

Application of AR-M961, a nonselective galanin agonist (20, 28), induced internalization of both GalR1–EGFP (Fig. 1*C*) and GalR2–EGFP (Fig. 1*G*). However, AR-M1896, a GalR2 (and -R3) agonist (28, 29), caused internalization of GalR2–EGFP (Fig. 1*H*) but not of GalR1–EGFP (Fig. 1*D*). Similar results were obtained in HEK293 cells (data not shown). Preincubation with the putative galanin antagonists M35 (100 nM) or M40 (100 nM) (30) did not prevent galanin-induced internalization of GalR1–EGFP (data not shown). In fact, both M35 and M40 induced internalization of GalR1–EGFP by themselves (data not shown).

Functional Activity of GalR1–EGFP Expressed in PC12 Cells. It has been reported that GalR1 mediates galanin-induced inhibition of
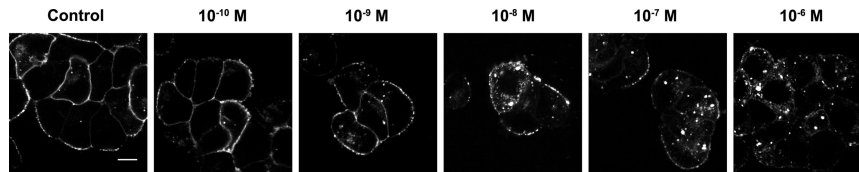
NEUROSCIENCE

Fig. 2. Dose-dependent, galanin-induced internalization of GalR1–EGFP in transfected PC12 cells. Cells were treated for 30 min at 37°C with increasing concentrations of galanin and examined with confocal microscopy. A distinct internalization is seen at 10^{-8} M. (Scale bar: 5 μm.)

forskolin-stimulated adenylate cyclase activity and thus of cAMP formation (31–33). To ensure that the EGFP-tagged GalR1 could transduce a physiological signal, we measured the effect of galanin on forskolin-stimulated accumulation of cAMP in stably transfected and untransfected PC12 cells. Galanin at 100 nM reduced the forskolin-stimulated cAMP accumulation to $68 \pm 5\%$ (mean $\pm$ SE; $n = 5$) as compared with controls without galanin ($P < 0.05$). Galanin did not alter forskolin-stimulated cAMP formation in naïve PC-12 cells without GalR1-EFGP ($101 \pm 8\%$; $n = 4$).

GalR1–EGFP Internalization via the Clathrin-Dependent Endocytic Pathway. When transfected cells were treated with Texas red-conjugated transferrin (20 μg/ml), this clathrin pathway marker was located in cytoplasmic vesicles (Fig. 3*Ac*), clearly separated from the membrane-bound GalR1–EGFP (Fig. 3 *Aa* and *Ab*). After addition of galanin at 100 nM, internalized GalR1–EGFP was colocalized with transferrin (compare Fig. 3 *Ad–Af* with 3 *Aa–Ac*). The internalization was blocked by prior incubation in 0.4 M sucrose for 5 min (data not shown), a hypertonic treatment known to inhibit clathrin-dependent receptor internalization by blocking clathrin-coated pit formation (34). To more specifically address the role of clathrin in GalR1 endocytosis, we also knocked down clathrin expression by using siRNAs. Clathrin staining in controls was cytoplasmic (Fig. 3 *Ah* and *Ai*) vs. the membrane-bound GalR1–EGFP (Fig. 3*Ag*). Although only ≈10–15% of the cells were successfully transfected with clathrin siRNA, these cells showed a much lower level of clathrin staining, and GalR1–EGFP internalization induced by galanin (100 nM) was much impaired in such cells (Fig. 3 *Aj–Al*). In contrast, the GalR1–EGFP was completely internalized in adjacent, untransfected cells with strong clathrin staining (Fig. 3 *Aj–Al*). These data further support the notion that GalR1–EGFP trafficking utilizes the clathrin-dependent endocytic pathway.

Lysosomal Sorting of the Endocytosed GalR1–EGFP. After incubation with galanin for 10 min, the intensity of intracellular GalR1–EGFP was increased by $42 \pm 11\%$ (mean $\pm$ SE; $n = 39$; $P < 0.05$) (Fig. 4*A*) as compared with basal conditions but returned to prestimulation level ($-3 \pm 5\%$; $n = 23$) after prolonged stimulation (45 min) (Fig. 4*A*). The intensity of fluorescence at the plasma membrane was also reduced, by $90 \pm 1\%$ ($n = 22$) (Fig. 4*B*), after galanin stimulation for 45 min. These results indicate an actual reduction of GalR1–EGFP, perhaps attributable to degradation. To address this question, cells were incubated with LysoTracker, a lysosomal marker, for 45 min at 37°C, with and without galanin treatment. Under baseline conditions, Lyso-Tracker was located in large vesicles in the cytoplasm (Fig. 3 *Bb* and *Bc*), whereas GalR1–EGFP was predominantly located at the cell surface (Fig. 3 *Ba* and *Bb*). After coincubation with galanin for 45 min, most of the GalR1–EGFP was internalized (Fig. 3 *Bd* and *Be*), and many of the GalR1–EGFP-positive structures colocalized with LysoTracker ($54 \pm 5\%$; $n = 15$) (Figs. 3 *Be* and *Bf* and 5). Taken together, these data suggest that a significant proportion of internalized GalR1 is transported into lysosomes for degradation.

Lysosomal-Targeting Motif (YXXØ) at the C-Terminal of GalR1. To identify a specific signal in GalR1 responsible for the targeting to the late endosomal/lysosomal compartment, we performed an interspecies sequence alignment of the N- and C-terminal regions of GalR1. We found that GalR1 contains a completely
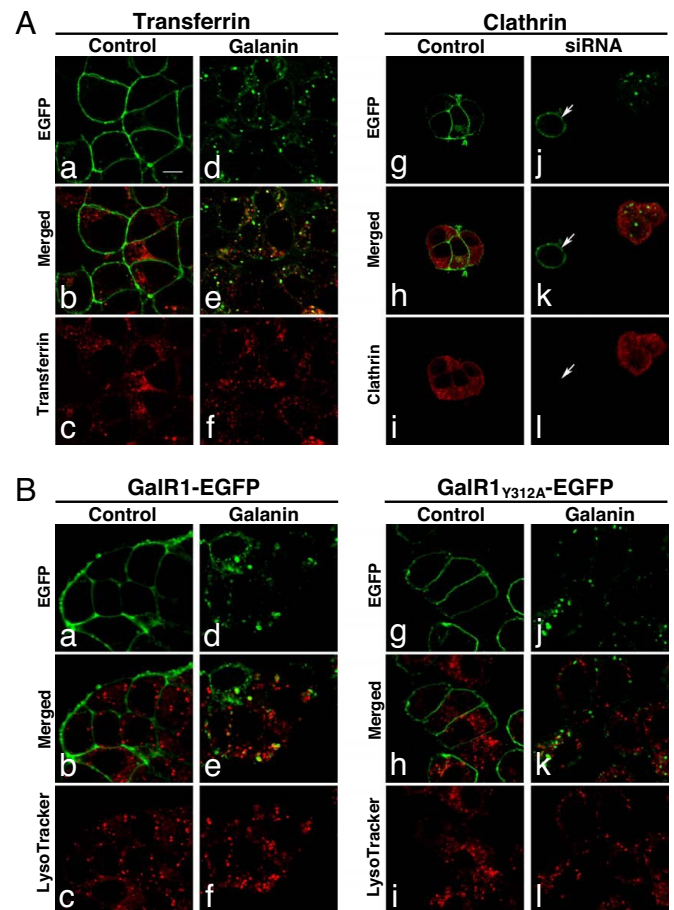

Fig. 3. GalR1 is internalized via the clathrin-dependent pathway and targeted to lysosomes by the YXXØ motif. (*Aa–Af*) Colocalization of GalR1–EGFP with transferrin. Transfected PC12 cells were incubated with Texas Red-transferrin alone (*Aa–Ac*) or with galanin (*Ad–Af*) for 30 min. Colocalization of GalR1–EGFP with transferrin (yellow) can be observed in the merged images. (*Ag–Al*) siRNA-mediated clathrin-silencing impairs galanin-induced GalR1–EGFP internalization. Clathrin was labeled with rhodamine Red-X (red). Double-fluorescence staining of GalR1–EGFP PC12 cells without siRNA (*Ag–Ai*) or treated with siRNA (*Aj–Al*). Transfected cells were incubated with galanin for 15 min (*Aj–Al*). Note the lack of internalization in cells transfected with siRNA (arrow) vs. internalization on clathrin-positive cells. (*Ba–Bl*) Colocalization of GalR1–EGFP (*Ba–Bf*) or GalR1$_{Y312A}$–EGFP (*Bg–Bl*) with LysoTracker. Transfected cells were incubated with LysoTracker alone for 45 min (*Ba–Bc* and *Bg–Bi*), or plus galanin (*Bd–Bf* and *Bj–Bl*). Note colocalization of the two markers (yellow) in cells with naïve GalR1 (*Be*) vs. lack of colocalization in cells with mutated GalR1 (*Bk*). [Scale bars: 5 μm (*Aa–Af* and *Ba–Bl*); 8 μm (*Ag–Al*).]
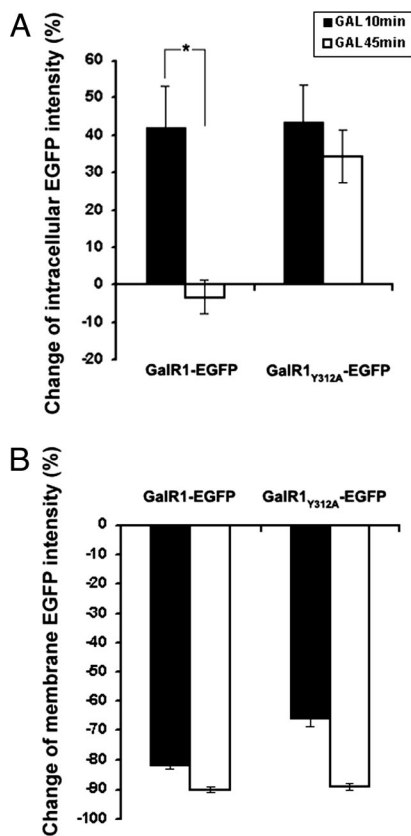
Fig. 4. Replacement of tyrosine by alanine results in a marked reduction of degradation of GalR1–EGFP. PC12 cells transfected with GalR1–EGFP (WT) or GalR1$_{Y312A}$–EGFP (Y312A) were incubated with galanin for 10 min (filled) or 45 min (open) at 37°C. (*A*) Change of intracellular EGFP intensity after galanin stimulation with different incubation time. (*B*) Change of membrane EGFP intensity after galanin stimulation with different incubation time. *, $P < 0.05$ for 10 min vs. 45 min.

conserved membrane-proximal YXXØ (where Ø is a bulky hydrophobic residue and X any amino acid) at the C terminus (Fig. 6), a motif implicated in endosome/lysosome targeting of diverse proteins (35). We mutated the acidic residue Y in YXXØ to A, because the Y in the YXXØ motif previously has been shown to be important for the lysosomal sorting (35). Thus, a GalR1$_{Y312A}$–EGFP mutant was created and stably expressed in PC12 cells. These cells were incubated with LysoTracker for 45 min at 37°C with and without galanin treatment. In the absence of galanin, GalR1$_{Y312A}$–EGFP mutant was located on the plasma membrane (Fig. 3*Bg*), whereas LysoTracker was distributed in the cytoplasm (Fig. 3*Bi*), the two markers, thus, being clearly separated (Fig. 3*Bh*). Galanin at 100 nM induced internalization of the GalR1$_{Y312A}$–EGFP mutant. Similar to WT GalR1–EGFP, the intensity of intracellular GalR1$_{Y312A}$–EGFP was increased by 44 ± 10% (mean ± SE; $n = 24$; $P < 0.05$) (Fig. 4*A*) compared with prestimulation level. However, it remained at a similar level (34 ± 7%; $n = 23$) after prolonged stimulation (45 min) (Fig. 4*A*), whereas the intensity of fluorescence at the plasma membrane was reduced by 89 ± 1% ($n = 22$) (Fig. 4*B*). Moreover, there was only minimal overlap between GalR1$_{Y312A}$–EGFP (Fig. 3*Bj*) and LysoTracker (Fig. 3*Bl*) (22 ± 3%; $n = 11$) (Fig. 5), although both of them were located in the cytoplasm (Fig. 3*Bk*). These findings suggest that GalR1$_{Y312A}$–EGFP is not preferentially delivered to lysosomes, in contrast to the WT GalR1–EGFP, and that lysosomal sorting of the endocytosed GalR1–EGFP may be mediated through tyrosine motif Y̧XXØ at the C terminus of the receptor.
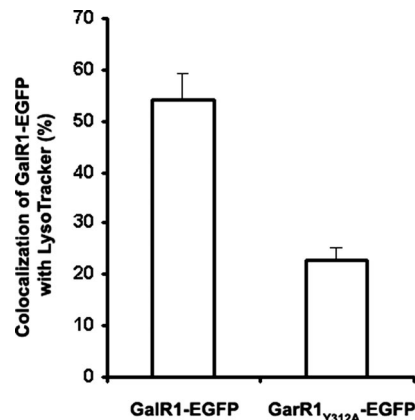

Fig. 5. Replacement of tyrosine by alanine reduces colocalization of GalR1–EGFP with LysoTracker. PC12 cells transfected with GalR1–EGFP (WT) or GalR1$_{Y312A}$–EGFP (Y312A) were incubated with galanin for 45 min at 37°C. *, $P < 0.05$ for GalR1–EGFP vs. GalR1$_{Y312A}$–EGFP.

Discussion

The present results show that a GalR1–EGFP receptor fusion protein, expressed at the plasma membrane of both PC12 and HEK-293 cells, is functional and endocytosed after galanin stimulation. Thus, these cells are suitable tools for analyzing the subcellular distribution and trafficking of the receptor. This is in agreement with a recent study by Wirz *et al.* (25) on CHO cells transfected with a GalR1–CFP or –YFP construct. By using time-lapse confocal imaging and fluorescence resonance energy transfer, Wirz *et al.* have observed a dose-dependent, GalR1-selective, forskolin-sensitive internalization, as well as a substantial homodimerization of GalR1 on the cell surface (25).

Several galanin receptor ligands were studied with the GalR1–EGFP chimera. ARM-961, a nonselective galanin receptor agonist (28), induced internalization of both GalR1–EGFP and GalR2-GFP, whereas the GalR2 (R3) agonist AR-M1896 (28, 29) failed to do this. Neither M35 nor M40, two chimeric peptides and putative galanin antagonists (30), prevented galanin-induced internalization. In fact, both M35 and M40 elicited internalization of GalR1–EGFP, in agreement with studies on PC12 cells transfected with GalR2 (27). However, it has also been reported that administration of M40 does not induce GalR1 internalization in CHO cells (25).

Studies with Texas Red-conjugated transferrin indicated that EGFP–GalR1 internalization involves the clathrin endocytic pathway (36). This was further supported by the fact that prior incubation in 0.4 M sucrose, a hypertonic medium known to cause abnormal clathrin polymerization (34), blocked this internalization. Moreover, we directly addressed this issue by using siRNA to down-regulate clathrin expression. Only some PC12 cells showed strong inhibition of clathrin synthesis, but, in these cases, GalR1 internalization was blocked. Thus, these data suggest that GalR1 receptors undergo ligand-induced, clathrin-dependent internalization.

It is well known that the internalized receptors can be dephosphorylated and recycle back to the cell surface and reinsert into the membrane (37), as also shown for GalR2 (27). Alternatively the internalized receptors are directed to lysosomes and into the degradation pathway, particularly in the case of prolonged receptor stimulation (37). The intensity of intracellular GalR1–EGFP was increased by 42% at 10 min after galanin stimulation, but at 45 min there was a decrease compared with the prestimulation level, accompanied by a continued loss of membrane-associated receptor, suggesting degradation. The colocalization of GalR1–EGFP and the lysosomal marker Lyso-

NEUROSCIENCE

Fig. 6. Sequence alignment of the GalR1 C terminus from five different species, with absolutely conserved YXXØ motif, and of rat GalR2. Abbreviations, boundaries, and accession numbers for the motifs are as follows: Hs, *Homo sapiens* (residues 314–317; NCBI accession no. NP_001471); Pt, *Pan troglodytes* (residues 314–317, NCBI accession no. XP_523975); Cf, *Canis familiaris* (residues 316–319; NCBI accession no. XP_541048); Mm, *Mus musculus* (residues 313–316; NCBI accession no. NP_032108); Rn, *Rattus norvegicus* (residues 314–317; NCBI accession no. NP_037090).

Tracker after prolonged galanin stimulation indicates that a significant quantity of internalized GalR1 is transported to lysosomes for degradation. This is in agreement with the view that the long-term desensitization seen after galanin-induced hyperpolarization in noradrenergic locus coeruleus neurons is mediated by GalR1 (21).

Increasing evidence suggests that targeting signals of an internalized GPCR receptor either to recycling or lysosomal degradation pathways may reside in its cytoplasmic tail (35, 38). Most signals consist of short, linear peptide sequences including tyrosine-based sorting signals (such as NPXY and XYYØ motifs), di-leucine-based signals such as ([DE]XXX-L[LI] and DDXXLL motifs) and PDZ ligands. All of these signals are recognized by adaptor-protein complexes and mediate endosomal sorting of GPCRs (35, 38). It has also been shown that GPCR ubiquitination regulate receptor trafficking (35, 38). Thus, diverse pathways and multiple complex mechanisms exist for endocytic sorting of GPCRs. YXXØ is present in the cytoplasmic tail of many integral proteins of the mammalian plasma membrane, including GPCRs and has multiple functional roles, including endocytosis, lysosomal targeting and sorting of protein to the basolateral plasma membrane (35, 38). It has been suggested that YXXØ is involved in rapid internalization and lysosome sorting of GPCRs, depending on its position within the C terminus. Thus, purely endocytic YXXØ signals mostly are present at residues 10–40 from the transmembrane domain, whereas lysosomal-targeting YXXØ are closer, that is at residues 6–9 (35). For example, protease-activated receptor-1 has two YXXØ motifs in its C-terminal. Whereas the distal YKKL motif controls constitutive internalization, the proximal YSIL motif regulates lysosomal sorting (38). On the other hand, kinin B_1 and B_2 receptors have C-terminal YXXØ motifs located at the 15th residue from the transmembrane domain. Both receptors undergo ligand-induced internalization, but whereas the B_2 receptor is recycled, B_1 is degraded (39).

Our interspecies GalR1 sequence alignment revealed the presence of a completely conserved YXXØ motif at the seventh residue from the transmembrane domain, indicating a lysosomal-targeting signal. In fact, replacement of Y by A resulted in a marked reduction of delivery to lysosomes, suggesting an important role of the C-terminal YXXØ motif in postendocytotic trafficking of GalR1. In contrast, GalR2 does not have the YXXØ motif in its C terminus (Fig. 6) and mainly recycles back to the cell membrane after washout of galanin (27).

Materials and Methods

Construction of the EGFP-Tagged GalR1 Receptor. The ORF of the rat GalR1 gene was reversely transcribed and amplified with a Tian one tube RT-PCR kit (Roche) from rat brain total RNA by using 5′-CCCAAGCTTCCACCATGGAACT-GGCTCCGGTGAACCTCAGTGAAGGG-3′ (forward) and 5′- GATCCGCG-GCACGTGGGTGCAGTTGGTGGAG-3′ (reverse) primers. This PCR product was ligated into pEGFP-Nl vector (Clontech Laboratories) by using HindIII and SacII

to give rise to a GalR1–EGFP expression vector. A GalR1$_{Y312A}$ mutant was created by site-directed mutagenesis to substitute tyrosine for alanine at amino acid 312 by using the QuikChange mutagenesis kit (Stratagene) and according to the instructions of the manufacturer. The plasmids of interest were identified by restriction mapping and DNA sequencing.

Stable Transfection of PC12 and HEK293 Cells. Cells were transfected by using 1 μg of DNA and employing the Effectene Transfection Reagent kit (Qiagen). To generate stable transfectants, the cells were selected in the presence of the antibiotic Geneticin (G418, Sigma) at 800 μg/ml. Clone expression was initially examined by fluorescence microscopy, and clones for further study were selected and expanded. Transfected cells were starved for 4 h before experiments.

Analysis of GalR1–EGFP Internalization and Trafficking. For ligand-induced internalization studies, the cells were treated with galanin (Bachem), ARM961, ARM1896, M35, and M40 (all at 100 nM) for 10 or 45 min at 37°C unless otherwise indicated. Some cells were coincubated with galanin (100 nM) and Texas Red transferrin (20 μg/ml, Sigma) or LysoTracker (1×10^{-6} M, Sigma) for 10 or 45 min at 37°C. Then the cells were fixed with 4% paraformaldehyde for 20 min at 4°C. Images were acquired by using a laser scanning confocal system (Radiance Plus, Bio-Rad). For dual-color analysis, images were collected sequentially in single-channel mode. For quantification of EGFP fluorescence, the digital image analysis was performed to measure the subcellular distribution of the GalR1–EGFP by using the NIH Image program (version 1.6). Thus, the mean fluorescence density of EGFP of whole cell body, membrane (Fdm), cytoplasm (Fdc), and nucleus was measured, and the Fdm/Fdc ratio was calculated. Statistical analyses were performed by using Student's t test, and the difference was considered significant at $P < 0.05$.

Measurements of cAMP. The cAMP [^{125}I] Direct Biotrak Scintillation Proximity Assay (SPA) System (dual range) (Amersham Biosciences) was used for determination of cAMP. Briefly, after harvesting and washing with PBS and incubation at 37°C in Hepes buffer, the cells were resuspended in a 96-well culture plate (3×10^4 cells/well) in the presence or absence of galanin and incubated with 0.3 mM forskolin at 37°C for 15 min, followed by the addition of 10% dodecyltrimethylammonium bromide to stop the reaction. The cells were lysed, and total cAMP was assayed according to a nonacetylation protocol. The samples were counted by using a Trilux 1450 Micro beta counter (Wallac), and the data were analyzed by using Prism software (GraphPad).

siRNA Knockdowns. siRNA knockdown of endogenous clathrin was carried out according to the Santa Cruz siRNA Transfection Protocol (Santa Cruz Biotechnology). Briefly, PC12 cells stably expressed with GalR1–EGFP, grown to 50–80% confluence in a 24-well plate, were transfected with Clathrin LCB siRNA (sc-37024, Santa Cruz Biotechnology) according to the protocol. After incubating the cells for 5–7 h at 37°C in a CO_2 incubator, the transfection mixture was removed and replaced with 1× normal growth medium. The experiment was carried out 72 h after transfection. An anti-clathrin antibody (sc-12735, Santa Cruz Biotechnology) was used at a dilution of 1:400 to assay efficiency of knockdown.

1. Tatemoto K, Rökaeus A, Jörnvall H, McDonald TJ, Mutt V (1983) Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett* 164:124–128.
2. Crawley JN (1996) Minireview. Galanin-acetylcholine interactions: Relevance to memory and Alzheimer's disease. *Life Sci* 58:2185–2199.
3. Ögren SO, *et al.* (1998) Modulation of acetylcholine and serotonin transmission by galanin: Relationship to spatial and aversive learning. *Ann NY Acad Sci* 863:342–363.
4. Mufson EJ, Counts SE, Perez SE, Binder L (2005) Galanin plasticity in the cholinergic basal forebrain in Alzheimer's disease and transgenic mice. *Neuropeptides* 39:233–237.
5. Fuxe K, *et al.* (1998) Galanin modulates 5-hydroxytryptamine functions: Focus on galanin and galanin fragment/5-hydroxytryptamine$_{1A}$ receptor interactions in the brain. *Ann NY Acad Sci* 863:274–290.
6. Weiss JM, Bonsall RW, Demetrikopoulos MK, Emery MS, West CH (1998) Galanin: A significant role in depression? *Ann NY Acad Sci* 863:364–382.
7. Barrera G, *et al.* (2005) One for all or one for one: Does co-transmission unify the concept of a brain galanin ''system'' or clarify any consistent role in anxiety? *Neuropeptides* 39:289–292.
8. Ögren SO, Kuteeva E, Hökfelt T, Kehr J (2006) Galanin receptor antagonists: A potential novel pharmacological treatment for mood disorders. *CNS Drugs* 20:633–654.
9. Lu X, Sharkey L, Bartfai T (2007) The brain galanin receptors: Targets for novel antidepressant drugs. *CNS Neurol Disord Drug Targets* 6:183–192.
10. Wiesenfeld-Hallin Z, Xu XJ (2001) Neuropeptides in neuropathic and inflammatory pain with special emphasis on cholecystokinin and galanin. *Eur J Pharmacol* 429:49–59.
11. Liu HX, Hökfelt T (2002) The participation of galanin in pain processing at the spinal level. *Trends Pharmacol Sci* 23:468–474.
12. Kalra SP, Horvath TL (1998) Neuroendocrine interactions between galanin, opioids, and neuropeptide y in the control of reproduction and appetite. *Ann NY Acad Sci* 863:236–240.
13. Leibowitz SF (2005) Regulation and effects of hypothalamic galanin: Relation to dietary fat, alcohol ingestion, circulating lipids and energy homeostasis. *Neuropeptides* 39:327–332.
14. Holmes FE, Mahoney SA, Wynick D (2005) Use of genetically engineered transgenic mice to investigate the role of galanin in the peripheral nervous system after injury. *Neuropeptides* 39:191–199.
15. Branchek TA, Smith KE, Gerald C, Walker MW (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21:109–117.
16. O'Donnell D, Ahmad S, Wahlestedt C, Walker P (1999) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: Distinct distribution from GALR1. *J Comp Neurol* 409:469–481.
17. Seutin V, Verbanck P, Massotte L, Dresse A (1989) Galanin decreases the activity of locus coeruleus neurons *in vitro*. *Eur J Pharmacol* 164:373–376.
18. Sevcik J, Finta EP, Illes P (1993) Galanin receptors inhibit the spontaneous firing of locus coeruleus neurones and interact with mu-opioid receptors. *Eur J Pharmacol* 230:223–230.
19. Pieribone VA, *et al.* (1995) Galanin induces a hyperpolarization of norepinephrine-containing locus coeruleus neurons in the brainstem slice. *Neuroscience* 64:861–874.
20. Ma X, *et al.* (2001) Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res* 919:169–174.
21. Xu ZQ, Zheng K, Hökfelt T (2005) Electrophysiological studies on galanin effects in brain–progress during the last six years. *Neuropeptides* 39:269–275.
22. Drake MT, Shenoy SK, Lefkowitz RJ (2006) Trafficking of G protein-coupled receptors. *Circ Res* 99:570–582.
23. Dupre DJ, Hebert TE (2006) Biosynthesis and trafficking of seven transmembrane receptor signalling complexes. *Cell Signal* 18:1549–1559.
24. Wang S, Clemmons A, Strader C, Bayne M (1998) Evidence for hydrophobic interaction between galanin and the GalR1 galanin receptor and GalR1-mediated ligand internalization: Fluorescent probing with a fluorescein-galanin. *Biochemistry* 37:9528–9535.
25. Wirz SA, Davis CN, Lu X, Zal T, Bartfai T (2005) Homodimerization and internalization of galanin type 1 receptor in living CHO cells. *Neuropeptides* 39:535–546.
26. Xia S, *et al.* (2005) Constitutive and ligand-induced internalization of EGFP-tagged galanin R2 and RI receptors in PC12 cells. *Neuropeptides* 39:173–178.
27. Xia S, *et al.* (2004) Visualization of a functionally enhanced GFP-tagged galanin r2 receptor in pc12 cells: Constitutive and ligand-induced internalization. *Proc Natl Acad Sci USA* 101:15207–15212.
28. Liu HX, *et al.* (2001) Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: Selective actions via GalR1 and GalR2 receptors. *Proc Natl Acad Sci USA* 98:9960–9964.
29. Lu X, Lundström L, Bartfai T (2005) Galanin (2–11) binds to GalR3 in transfected cell lines: Limitations for pharmacological definition of receptor subtypes. *Neuropeptides* 39:165–167.
30. Bartfai T, Fisone G, Langel Ü (1992) Galanin and galanin antagonists: Molecular and biochemical perspectives. *Trends Pharm Sci* 13:312–317.
31. Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux JF (1994) Molecular cloning of a functional human galanin receptor. *Proc Natl Acad Sci USA* 91:9780–9783.
32. Parker EM, *et al.* (1995) Cloning and characterization of the rat GALR1 galanin receptor from Rin14B insulinoma cells. *Mol Brain Res* 34:179–189.
33. Burgevin MC, Loquet I, Quarteronet D, Habert-Ortoli E (1995) Cloning, pharmacological characterization, and anatomical distribution of a rat cDNA encoding for a galanin receptor. *J Mol Neurosci* 6:33–41.
34. Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* 108:389–400.
35. Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* 72:395–447.
36. Cao TT, Mays RW, von Zastrow M (1998) Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem* 273:24592–24602.
37. von Zastrow M (2001) Role of endocytosis in signalling and regulation of G-protein-coupled receptors. *Biochem Soc Trans* 29:500–504.
38. Marchese A, Paing MM, Temple BR, Trejo J (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol* 48:601–629.
39. Enquist J, Skröder C, Whistler JL, Leeb-Lundberg LM (2007) Kinins promote b2 receptor endocytosis and delay constitutive b1 receptor endocytosis. *Mol Pharmacol* 71:494–507.

NEUROSCIENCE