Constitutive Traffic of Melanocortin-4 Receptor in Neuro2A Cells and Immortalized Hypothalamic Neurons*

Received for publication, August 30, 2006, and in revised form, November 24, 2006. Published, JBC Papers in Press, December 12, 2006, DOI 10.1074/jbc.M608283200

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Melanocortin-4 receptor (MC4R) is a G protein-coupled receptor (GPCR) that binds α-melanocyte-stimulating hormone (α-MSH) and has a central role in the regulation of appetite and energy expenditure. Most GPCRs are endocytosed following binding to the agonist and receptor desensitization. Other GPCRs are internalized and recycled back to the plasma membrane constitutively, in the absence of the agonist. In unstimulated neuroblastoma cells and immortalized hypothalamic neurons, epitope-tagged MC4R was localized both at the plasma membrane and in an intracellular compartment. These two pools of receptors were in dynamic equilibrium, with MC4R being rapidly internalized and exocytosed. In the absence of α-MSH, a fraction of cell surface MC4R localized together with transferrin receptor and to clathrin-coated pits. Constitutive MC4R internalization was impaired by expression of a dominant negative dynamin mutant. Thus, MC4R is internalized together with transferrin receptor by clathrin-dependent endocytosis. Cell exposure to α-MSH reduced the amount of MC4R at the plasma membrane by blocking recycling of a fraction of internalized receptor, rather than by increasing its rate of endocytosis. The data indicate that, in neuronal cells, MC4R recycles constitutively and that α-MSH modulates MC4R residency at the plasma membrane by acting at an intracellular sorting step.

MC4R² belongs to a family of GPCRs that are expressed in the hypothalamic paraventricular nucleus as well at multiple sites such as the brain cortex, thalamus, hypothalamus, brainstem, and spinal cord (1, 2). Stimulation of MC4R by its natural agonist, α-MSH, is central to the control of food intake. In support of this, it has been reported that inactivation of MC4R in mice leads to obesity syndrome with hyperphagia, hyperglycemia, and hyperinsulinemia (3). In addition, intraventricular administration of α-MSH suppresses feeding (4). The importance of MC4R in food intake regulation is highlighted by the recent discovery that most of the known monogenetic causes of obesity are because of mutations in the MC4R (5–9). Binding of α-MSH to MC4R induces stabilization of the active conformation of the receptor. The activated receptor binds to Go, and this leads to stimulation of adenylyl cyclase and increased intracellular concentration of cAMP, which activates protein kinase A (10). Active protein kinase A initiates transcription of new genes by phosphorylating and activating cAMP-responsive element-binding protein.

Agonist-dependent phosphorylation of GPCRs leads to the formation of high affinity binding sites to which β-arrestins bind (11–13). In the classical pathway, binding of β-arrestins to GPCRs leads to uncoupling of the receptor from G proteins with consequent termination of the signal and β-arrestin-dependent recruitment of the clathrin adaptor protein complex-2 (AP-2). AP-2 binds to clathrin, and this leads to the formation of coated pits that includes GPCR. Nascent clathrin-coated vesicles containing GPCR are then pinched off by a process that requires association of a small GTPase, dynamin, at the neck of the pit (14). In agreement with this classical model of GPCR desensitization and internalization, it was found that exposure to α-MSH induces rapid internalization of GFP-tagged MC4R to endosomes in the human embryonic kidney 293 cells (HEK 293) (15, 16). Overexpression of dominant negative forms of dynamin1 and β-arrestin1 in these cells inhibited agonist-induced internalization of the MC4R (16). Exposure to agents that block clathrin-mediated endocytosis also inhibited agonist-induced internalization of MC4R (17). These data indicate that, in the HEK 293 cells, MC4R is internalized by a clathrin-dependent mechanism initiated by receptor binding to the agonist. Some GPCRs such as thyrotropin receptor (17), the yeast α-factor receptor (18), the CB1 cannabinoid receptor (19), and the protease-activated receptor-1 (20, 21) have been reported to undergo constitutive internalization also in the absence of the agonist. The cell distribution of MC4R has been mostly studied in HEK 293 cells that do not express endogenous
MC4R. Neuroblastoma Neuro2A (N2A) cells originate from the neural crest (22), have been reported to express endogenous MC4R mRNA, and to respond to α-MSH by enhanced neurite extension (23). GT1-7 are immortalized hypothalamic neurons derived from transgenic mice expressing the SV40 T-antigen oncogene under the control of the gonadotropin-releasing hormone promoter (24–26). GT1-7 cells do not express glial markers and express MC4R and other neuronal markers (16, 25, 26). We observed that both in unstimulated N2A and GT1-7 cells, a pool of GFP-tagged MC4R had an intracellular localization. This prompted us to determine whether MC4R is constitutively internalized and recycled in these cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Lipofectamine 2000 and SNAP-23 siRNA duplexes targeting position 401–419 (ccagugguggauuacg) were from Dharmacon, Inc. (Chicago, IL). Rabbit polyclonal SNAP-23 antibodies, mouse monoclonal SNAP-25 antibodies, and mouse monoclonal synaptobrevin-2 (VAMP-2) antibodies were purchased from Synaptic Systems (Goettingen, Germany); mouse monoclonal synaptobrevin-2 antibodies (clone HPC-1), iron-saturated transferrin (Tf), α-MSH, 3-isobutyl-1-methylxanthine (IBMX), forskolin, brefeldin A (BFA), and GFP antibodies were from Sigma; peroxidase (POD)-conjugated anti-hemagglutinin (HA) antibody (3F10), mouse monoclonal anti-HA antibody (12CA5), secondary POD-conjugated anti-mouse IgG, protease inhibitor mixture (Complete Mini), and 2',2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) tablets were from Roche Applied Science; POD-conjugated transferrin (TF-POD) and secondary POD-conjugated anti-rabbit IgG were from Pierce; tetramethylrhodamine-conjugated human Tf (TMR-Tf) was from Molecular Probes (Eugene, OR); FITC-conjugated anti-mouse IgG and Cy3-conjugated anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA); gold-conjugated anti-rabbit and anti-mouse IgG were from BBI International (Cardiff, UK); enhanced chemiluminescence detection kits were from PerkinElmer Life Sciences. Agouti-related protein (AGRP, 83-132) was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Enhanced green fluorescent protein expression vector pEGFP-N2 was from Clontech. pCB expression vector containing human dynamin 1 cDNA (DynWT-pCB) or the K44A mutant of human dynamin 1 (DynK44A-pCB) were a kind gift of Dr. Sandra Schmid, the Scripps Research Institute, La Jolla, CA. The plasmid pCDTR containing the HA-MC4R sequence upstream of, and in-frame with, the GFP coding sequence to express HA-MC4R-GFP. To generate HA-MC4R without GFP, PCR amplification was done by using the MC4R-pCMV-XL4 template, the same forward primer as above, and as reverse primer, CGGTGGATC-CCGGGGCTTAATATCTGCTAGACACTGCA, where the MC4R stop codon (boldface) was maintained. The purified PCR product was digested with HindIII and BamHI and ligated to HindIII-BamHI digested pEGFP-N2. The ATG codon of GFP was mutated by using QuickChange site-directed mutagenesis kit (Stratagene). Plasmid DNA for transfections into mammalian cells was isolated using the DNA purification system from Promega.

**Cell Culture, Transfection, and Silencing**—N2A and N2A expressing BonT/E (G14) cells were cultured in DMEM with 10% fetal bovine serum and penicillin/streptomycin. Hypothalamic GT1-7 cells were a kind gift of Dr. R. I. Weiner (University of California, San Francisco). GT1-7 cells were cultured in DMEM/F-12 50:50 with 10% fetal bovine serum, 15 mM HEPES, pH 7.4, and penicillin/streptomycin. N2A, G14, and GT1-7 cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 and following the manufacturer’s instructions. N2A cells stably expressing HA-MC4R-GFP were selected by growth with 1.2 mg/ml G 418. For SNAP-23 silencing in N2A and G14 cells, cells were transfected either with 200 nM SNAP-23 siRNA or with 200 nM nontargeting siRNA pool using Lipofectamine 2000. Tf and MC4R recycling was measured 48 h after the siRNA transfection. Prior to all experiments, cells were washed twice with DMEM without serum and incubated for 16 h in the same medium.

**Gel Electrophoresis and Immunoblotting**—Separation of proteins by SDS-PAGE, immunoblotting with the indicated primary antibodies, and secondary POD-conjugated antibodies using enhanced chemiluminescence detection, densitometry, and protein determination were performed as described (27).

**Fluorescence Microscopy**—To visualize the distribution of HA-MC4R-GFP, N2A cells transiently transfected with HA-MC4R-EFGP were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. Images were captured using CARV spinning confocal imaging system attached to an Olympus X-71 fluorescence microscope. Images were collected by using a Photometrics CoolSnap HQ camera controlled by IP lab software (Scanalytics, Inc, Fairfax, VA). To visualize the distribution of MC4R and TR at the cell surface, N2A cells transiently expressing HA-MC4R and exogenous human TR were washed three times with DMEM, incubated in the same medium for 1 h at 37 °C, transferred in ice, and incubated with DMEM containing TMR-Tf (5 μg/ml) and anti-HA antibodies for 1 h. Cells were washed with PBS, fixed as above, and incubated with PBS containing 100 μg/ml ovalbumin and...
FITC-conjugated anti-mouse IgG without addition of detergents. Cells were visualized by total internal reflection (TIRF) microscopy by using an Olympus 1X71 inverted microscope equipped with argon laser (488 nm) and helium/neon laser (543 nm). Images were collected by using Metamorph software (Molecular Devices, Sunnyvale, CA). To study the localization of MC4R in respect to internalized TR, N2A cells transiently expressing HA-MC4R-GFP and treated as above were incubated with 5 μg/ml TMR-Tf at 37 °C for 1 h. Cells were washed and fixed, and images were captured with the confocal microscope as described above. Antibody-feeding experiments were done with N2A and GT1-7 cells transiently expressing HA-MC4R-GFP incubated for 1 h either at 4 °C or at 37 °C in DMEM with anti-HA mouse monoclonal antibodies (clone 12CA5). Cells were washed with PBS, fixed with formaldehyde, and further processed for secondary antibody staining as described (28) with Cy3-conjugated anti-mouse IgG in PBS containing 100 μg/ml ovalbumin without (nonpermeabilized cells) or with (permeabilized cells) 0.2% Triton X-100. To observe the localization of internalized MC4R and TR, N2A cells transiently expressing HA-MC4R and human TR were incubated with anti-HA antibodies and 5 μg/ml TMR-Tf in DMEM at 37 °C for 1 h to endocytose TMR-Tf/TR and mouse anti-HA-HA-TR complexes. Cells were washed, fixed, permeabilized with PBS containing 0.2% Triton X-100, and stained with anti-mouse FITC antibodies. Images were captured by confocal microscopy as above.

**Immuno electron Microscopy**—For pre-embedding immunocytochemistry, broken agarose-embedded N2A cells expressing high levels of HA-MC4R-GFP were prepared as described before (28, 29) and probed with primary rabbit antibodies against the GFP N terminus (1:5 dilution), and secondary anti-rabbit IgG was conjugated to a 10 nm gold particle from BBI International (Cardiff, UK). For post-embedding immunocytochemistry, the N2A cells expressing HA-MC4R-GFP grown on coverslips were fixed with PBS containing 4% paraformaldehyde for 30 min at room temperature. Cell monolayers were sequentially dehydrated in ascending alcohols and infiltrated for 4 h at room temperature with three changes of LR white acrylic resin, hard (Sigma). Coverslips were covered with a cylindrical capsule filled with fresh LR white resin and polymerized at 60 °C for 24 h. Thin sections were collected on 400-mesh nickel grids, incubated for 30 min at room temperature in TBS blocking buffer (20 mM Tris-HCl, pH 7.6, 225 mM NaCl, 1% BSA) containing 5% normal goat serum, and then incubated overnight at 4 °C with rabbit anti-GFP antibodies and monoclonal antibodies against clathrin heavy chain (clone TD.1, Pharmingen). After 10 min of washing in TBS blocking buffer, samples were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 20 nm diameter gold particles and with goat anti-mouse IgG conjugated to 10 nm diameter gold particles. Control grids were also included in which the primary antibody was omitted. Sections were stained with uranyl acetate and lead citrate.

**Quantification of HA-MC4R-GFP by Enzyme-linked Immunoassays**—To quantify HA-MC4R-GFP expression at the cell surface, N2A cells either transiently or stably expressing HA-MC4R-GFP were fixed with 3.7% formaldehyde for 30 min at room temperature, washed three times with PBS, and incubated with PBS containing 1% BSA for 30 min and then with the same medium containing POD-conjugated anti-HA antibodies (50 milliunits/ml) for 1 h. Mock-transfected cells were used as a control. Cells were washed with PBS three times, and POD activity was measured by incubating cells for 1 h at 37 °C with a water-soluble POD substrate (ABTS, 1 mg/ml) following the manufacturer’s instructions. The oxidized product was detected by reading its absorbance at 405 nm using a mQuant universal microplate spectrophotometer (Bio-Tek Instruments, Inc.). To detect endogenous MC4R in murine N2A cells, and to estimate the amount of total MC4R in N2A cells stably expressing HA-MC4R-GFP (human receptor), an immunoaassay was performed using an antibody against the extracellular N-terminal domain of mouse MC4R (amino acid 21–33 corresponding to YGLHSNASESLGK). The sequence of the human MC4R differs from that of the mouse by one amino acid substitution (Gly to Arg) at position 22. The cells were washed with PBS and fixed in 3.7% formaldehyde at room temperature. Cells were incubated with PBS containing 1% BSA followed by incubation with the anti-MC4R antibody (400 ng/ml) for 1 h. Cells were washed with PBS six times and incubated with POD-conjugated anti-rabbit secondary antibodies for 1 h. Cells were washed six times with PBS and incubated with ABTS substrate for 1 h at 37 °C. The oxidized product was detected as described above.

To study the effect of α-MSH on the amount of MC4R at the cell surface, N2A cells stably expressing HA-MC4R-GFP were preincubated for 1 h at 37 °C with DMEM containing 100 μM cycloheximide to inhibit protein synthesis. The cells were then incubated in the continuous presence of cycloheximide with or without 100 nM α-MSH for different time points. Cells were fixed, and the cell surface MC4R was measured as described above. For the BFA experiments, cycloheximide-treated N2A cells stably transfected with HA-MC4R-GFP were incubated for 2 h at 37 °C with and without 10 μg/ml BFA in the continuous presence of cycloheximide. Cells were washed and fixed, and surface HA-MC4R-GFP was measured as above.

For the antibody-feeding experiments, cycloheximide-treated N2A cells either transiently or stably expressing HA-MC4R-GFP were incubated for 30 min at 4 °C in DMEM with 50 milliunits/ml POD-conjugated anti-HA antibodies in the presence of cycloheximide. Cells were then further incubated at 4 or 37 °C for 1 h in the continuous presence of the anti-HA antibodies and cycloheximide. Cells were transferred in ice, washed three times with PBS, fixed with formaldehyde, and washed twice with PBS. To measure total and cell surface HA-MC4R-GFP, POD activity was assayed by preincubating cells for 30 min at room temperature in PBS with (permeabilized cells) and without (unpermeabilized cells) 0.2% Triton X-100, respectively, prior to addition of the ABTS substrate. For some antibody-feeding experiments, anti-HA antibodies bound to cell surface HA-MC4R-GFP were stripped off by incubation for 5 min at 4 °C in DMEM adjusted to pH 2.0 prior to washing with PBS and fixation with formaldehyde. For these experiments, antibody-labeled HA-MC4R-GFP was measured after incubation with PBS containing 0.2% Triton X-100 for 30 min to permeabilize cells prior to addition of the POD substrate.
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To study the rate of MC4R internalization, N2A cells stably expressing HA-MC4R-GFP were incubated with POD-conjugated anti-HA antibody at 4 °C to label the cell surface receptor. Cells were washed with DMEM and transferred at 37 °C to allow endocytosis of the receptor. At different time points cells were washed with PBS and fixed with formaldehyde. The amount of total and cell surface HA-MC4R-GFP was measured as described above. To study the effect of overexpression of wild-type and mutated dynamin 1 on the rate of internalization, N2A cells were transfected with HA-MC4R-GFP and co-transfected either with empty vector (pCB) or with wild-type dynamin 1 (Dyn-pCB) or with mutant dynamin (DynK44E-pCB). Twenty four hours after transfection, the internalization rate of MC4R was measured as above. To measure the effect of α-MSH on the rate of HA-MC4R-GFP endocytosis, cells were incubated with or without 100 nM α-MSH for 30 min at 4 °C. POD-conjugated anti-HA antibodies were added to the medium, and cells were further incubated at 4 °C to label HA-MC4R-GFP at the cell surface. Cells were washed, transferred at 37 °C in the absence and in the presence of α-MSH, and analysis of internalized HA-MC4R-GFP was carried out as described above. To study the rate of MC4R exocytosis, cycloheximide-treated N2A cells stably expressing HA-MC4R-GFP and GT1-7 cells transiently expressing the tagged receptor were incubated at 4 °C for 1 h with POD-conjugated anti-HA antibody in the continuous presence of cycloheximide. Cells were transferred to 37 °C, incubated for the different time points, washed, and fixed with formaldehyde. Surface and total HA-MC4R-GFP was measured as described above.

Measurement of Tf Uptake and Release—Cells plated in 35-mm dishes were washed with DMEM and incubated with DMEM for 1 h and further incubated with 8 µg/ml POD-Tf in DMEM for 1 h. Under these conditions, cells were maximally loaded with Tf. Cells were then washed with sodium citrate buffer (120 mM NaCl, 20 mM sodium citrate, pH 5.0) and PBS to remove unbound Tf-Pod as described by others (30, 31). Cells were then transferred to 37 °C and collected at different time points. Cells were scraped with 0.8 ml of PBS containing 0.5% Triton X-100 and incubated at 4 °C for 20 min. Cell lysates were cleared by centrifugation at 10,000 × g in an Eppendorf centrifuge. Pod activity in lysates (20 µl) was measured using the ABTS substrate.

Assay of cAMP—Cells were washed and incubated with Opti-MEM I containing 0.5 mM IBMX for 10 min and then stimulated with 100 nM α-MSH or with 10 µM forskolin for 15 min at 37 °C. The medium was aspirated, and cells were lysed in 0.1 N HCl. Intracellular cAMP was measured following the manufacturer’s instructions using the direct immunoassay kit from Biovision Research Products (Mountain View, CA).

Statistical Analysis and Curve Fitting—All experiments were done at least twice with triplicate samples. Data are expressed as mean ± S.D. of triplicate samples from a single experiment unless noted otherwise. For some experiments, data were compared by using the Student’s t test. Rate constants and plateaus of receptor internalization, externalization, and recycling were derived by fitting nonlinear regression models to the data, using the GraphPad Prism (GraphPad Software, San Diego, CA). The one-phase exponential decay model and the one-phase exponential association model for the internalization and externalization/recycling data, respectively, gave an R² > 0.95.

RESULTS

When N2A and GT1-7 cells were exposed to 100 nM α-MSH for 15 min, intracellular cAMP increased by ~10-fold (Fig. 1A), consistent with reports that both cell lines express endogenous MC4R. It has been found that attachment of an HA and a GFP tag to the N terminus and the C terminus of MC4R, respectively, does not change the receptor ability to bind to α-MSH or to respond to α-MSH stimulation by increasing intracellular cAMP levels (15, 16). We generated chimeric HA-MC4R-GFP to study the cell distribution and traffic of MC4R in the N2A and GT1-7 cells. In the N2A cells transiently transfected with the HA-MC4R-GFP plasmid, GFP fluorescence appeared at the plasma membrane and at an intracellular compartment (Fig. 1B). To detect HA-MC4R-GFP at the cell surface, we used an enzyme-linked immunoassay. Transiently transfected N2A cells were incubated at 4 °C with POD-conjugated monoclonal anti-HA antibodies as described by others (16). Plasma membrane-bound POD activity was measured by incubating the cells with the water-soluble POD substrate ABTS. POD activity was detected only in cells transiently transfected with the HA-MC4R-GFP plasmid and not in the control cells (Fig. 1C). Thus, the interaction of the POD-labeled anti-HA antibody with HA-MC4R-GFP is specific in our experimental conditions. We isolated a stably transfected N2A cell clone expressing HA-MC4R-GFP. The cell-associated POD activity in stably transfected cells was the same as in the transiently transfected cell clone (Fig. 1C). Also, the amount of exogenous MC4R detected with the antibody against the HA tag was the same in the transiently and stably transfected cells (Fig. 1D). Only 10% of transiently transfected cells expressed HA-MC4R-GFP (Fig. 1E). Thus, transiently transfected cells appeared to have ~10-fold more receptors per cell than the stably transfected cell clone. To determine whether this was the case, we measured the amount of HA-MC4R-GFP expressed in individual cells by fluorescence microscopy. In the population of transiently transfected cells expressing the receptor (n = 30), the fluorescence intensity of HA-MC4R-GFP was 6-fold (62% of transfected) when compared to the HA-MC4R-GFP plasmid and not in the control cells (Fig. 1F). In conclusion, these data suggest that the overall cell pool of MC4R in the stably transfected N2A cells is modestly increased by expression of the exogenous receptor.

The fluorescence distribution of HA-MC4R-GFP indicates that a fraction of the receptor is intracellular. To determine whether MC4R is constitutively internalized, we did antibody-
feeding experiments. For these experiments, live cells expressing HA-MC4R-GFP were incubated with anti-HA antibodies to monitor the receptor dynamics. Transiently and stably transfected N2A cells were incubated with POD-conjugated anti-HA antibodies at 37 °C, fixed, and either kept intact or permeabilized by the addition of 0.2% Triton X-100 to allow POD substrate entry in the cells. Permeabilized cells had ~2-fold more cell-associated POD activity that unpermeabilized cells (Fig. 2A). This suggests that the anti-HA-HA-MC4R-GFP complex at the plasma membrane is constitutively internalized during the 1-h incubation at 37 °C and that the fraction of intracellular receptors corresponds approximately to half of the total cell receptor pool both in the transiently and stably transfected cells. Transiently transfected N2A cells express 6-fold or more HA-MC4R-GFP than the stably transfected clone. These data indicate that, within the experimental system under study, a fraction of HA-MC4R-GFP is localized inside the cell independent of the level of receptor expression. To further investigate the possibility of an intracellular pool of HA-MC4R-GFP, transiently transfected N2A cells preincubated with POD-conjugated anti-HA antibodies were exposed to a medium buffered at pH 2.0. Under these conditions, antibody-antigen complexes at the cell surface dissociate, whereas internalized complexes are maintained (32). When incubation with the anti-HA antibodies was carried out at 4 °C, a temperature at which membrane traffic is blocked, cells lost virtually all of the associated POD activity when exposed to the acid medium. Thus, in this condition, anti-HA-HA-MC4R-GFP complexes were localized exclusively at the cell surface (Fig. 2B). When incubation of N2A cells with the antibody was carried out at 37 °C to allow for membrane traffic, the cell-associated POD activity was increased, and ~50% of the enzyme activity remained associated with the cells even after exposure to the low pH medium. This indicates that, at the elevated temperature, the antibody labels a larger population of receptor and that the anti-HA-HA-MC4R-GFP complexes are internalized. Similar results were obtained with the hypothalamic GT1-7 cells (Fig. 2C). These observations suggest that cell surface MC4R is endocytosed by N2A and GT1-7 cells. To image this, we carried out immunofluorescence experiments. A fraction of HA-MC4R-GFP labeled by incubation with anti-HA antibody at 4 °C was localized at the plasma membrane (Fig. 2D (panels i and ii), red fluorescence). Another pool of cell HA-MC4R-GFP, visualized by the GFP fluorescence, had no anti-HA staining consistent with its intracellular localization (Fig. 2D (panels i and ii), green fluorescence). When cells were incubated with the anti-HA antibodies at 37 °C, a fraction of the anti-HA-HA-MC4R-GFP complex was found in the intracellular compartment as indicated by the co-localization of the red and green fluorescence in the permeabilized cell sample (Fig. 2D (panel iv)). Similarly, when hypothalamic GT1-7 cells were incubated with the anti-HA antibody at 4 °C, cells had the anti-HA-HA-MC4R-GFP complex localized exclusively at the cell margins (Fig. 2E (panels i–iii)). When cells were incubated with the anti-
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body at 37 °C, the HA-HA-MC4R-GFP complex was found also at the intracellular localization (Fig. 2E (panels iv–vi)) indicating that MC4R is constitutively internalized.

Some GPCRs are constitutively internalized by clathrin-dependent endocytosis (20, 33). Other types of receptors, such as TR, are also internalized constitutively by clathrin-dependent endocytosis (34). We reasoned that if MC4R were constitutively internalized by a clathrin-dependent mechanism, the receptor would co-localize with transferrin and clathrin at the plasma membrane. To determine whether this is the case, N2A cells were co-transfected with HA-MC4R and human TR plasmids. TR at the surface of N2A cells was labeled by incubation with TMR-Tf at 4 °C and appeared as individual dots by TIRF microscopy (TMR-Tf; Fig. 3A (panel ii)), whereas HA-MC4R, labeled by anti-HA antibodies, had a more extensive punctate distribution (anti-HA; Fig. 3A (panel i)). Some MC4R puncta clearly co-localized with TR (Fig. 3A, arrows), whereas others did not (arrowheads). This indicates that at least a fraction of MC4R co-localizes with TR at the cell surface.

To visualize the plasma membrane localization of MC4R at the ultrastructural level, we used the stably transfected N2A cells expressing HA-MC4R-GFP. The GFP-tagged receptor appeared at the plasma membrane and in the intracellular localization virtually in all cells (Fig. 3B (panels i and ii)). Immunoelectron microscopy of the broken, agarose-embedded stably transfected N2A cells shows that HA-MC4R-GFP, labeled by anti-GFP antibodies, localized to coated pits at the plasma membrane (Fig. 3C (panels i and ii), arrows). No gold immunostaining was detected in parallel samples treated without primary antibodies (not shown). Post-embedding immunoelectron microscopy with anti-GFP (20 nm particle) and anti-clathrin antibodies (10 nm gold particles) showed co-localization of HA-MC4R-GFP and clathrin (Fig. 3C (panels iii–vi)). When N2A cells transiently expressing HA-MC4R-GFP were incubated with TMR-Tf, endocytosed Tf co-localized with a fraction of intracellular HA-MC4R-GFP (Fig. 3D). Moreover, when N2A cells transiently expressing HA-MC4R and exogenous TR were incubated at 37 °C with the anti-HA antibodies and TMR-Tf, a fraction of the internalized anti-HA-HA-MC4R complexes and Tf co-localized (Fig. 3D (panels iv–vi)). This shows that MC4R constitutive internalization to TR-containing endosomes is independent of the GFP tag. These experiments and those of Fig. 2 indicate that MC4R is constitutively internalized together with TR by clathrin-dependent endocytosis. A fraction of intracellular MC4R had a different distribution than TR (Fig. 3D, arrowheads) indicating that endocytosed MC4R localizes to other compartments in addition to those traversed by TR.

To determine the rate of MC4R internalization, we carried out antibody-feeding experiments using POD-conjugated antibodies against HA and stably transfected N2A cells expressing HA-MC4R-GFP. The anti-HA-HA-MC4R-GFP complexes disappeared from the cell surface with a 1/2 of 9.14 min (Fig. 4A). The amount of total cell anti-HA-HA-MC4R-GFP complexes, at the 0 and 60 min time points, remained the same (Fig. 4A). This indicates that MC4R constitutive internalization to TR-containing endosomes is independent of the GFP tag. These experiments and those of Fig. 2 indicate that MC4R is constitutively internalized together with TR by clathrin-dependent endocytosis. A fraction of intracellular MC4R had a different distribution than TR (Fig. 3D, arrowheads) indicating that endocytosed MC4R localizes to other compartments in addition to those traversed by TR.

FIGURE 2. MC4R is constitutively internalized in the absence of the agonist by N2A and GT1-7 cells. A, N2A cells either transiently or stably transfected with HA-MC4R-GFP and treated with cycloheximide were incubated with anti-HA-POD at 37 °C for 1 h, fixed under nonpermeabilizing (UP) conditions to measure MC4R at the cell surface, or permeabilized (P) to measure total cell MC4R by incubation with the POD substrate ABTS. B and C, N2A cells stably expressing HA-MC4R-GFP (B) or GT1-7 cells transiently transfected with HA-MC4R-GFP (C) were treated with cycloheximide and incubated either with POD-conjugated anti-HA antibodies at 4 or 37 °C for 1 h. Cells were washed with DMEM and treated for 5 min with DMEM adjusted to pH 2.0. Cells were washed and fixed, and POD activity was measured after cell permeabilization. D, N2A cells transiently transfected with HA-MC4R-GFP were incubated either at 4 °C (panels i and ii) or at 37 °C (panels iii and iv) in the presence of the mouse monoclonal antibody 12CA5 against the exofacial HA tag. Cells were washed and fixed, and POD activity was measured after cell permeabilization. E, GT1-7 cells transiently transfected with HA-MC4R-GFP were incubated with antibodies against the HA tag of MC4R either at 4 °C (panels i–iii) or at 37 °C (panels iv–vi), fixed, permeabilized, and stained with Cy3-conjugated secondary antibodies as above.

Expression of a dynamin1 mutant (dynamin1 K44A) that has partial rescue of the dynamin1 null mutant, showed that surface MC4R is internalized and not degraded. Some GPCRs are constitutively internalized by clathrin-dependent endocytosis (20, 33). Other types of receptors, such as TR, are also internalized constitutively by clathrin-dependent endocytosis (34). We reasoned that if MC4R were constitutively internalized by a clathrin-dependent mechanism, the receptor would co-localize with transferrin and clathrin at the plasma membrane. To determine whether this is the case, N2A cells were co-transfected with HA-MC4R and human TR plasmids. TR at the surface of N2A cells was labeled by incubation with TMR-Tf at 4 °C and appeared as individual dots by TIRF microscopy (TMR-Tf; Fig. 3A (panel ii)), whereas HA-MC4R, labeled by anti-HA antibodies, had a more extensive punctate distribution (anti-HA; Fig. 3A (panel i)). Some MC4R puncta clearly co-localized with TR (Fig. 3A, arrows), whereas others did not (arrowheads). This indicates that at least a fraction of MC4R co-localizes with TR at the cell surface. To visualize the plasma membrane localization of MC4R at the ultrastructural level, we used the stably transfected N2A cells expressing HA-MC4R-GFP. The GFP-tagged receptor appeared at the plasma membrane and in the intracellular localization virtually in all cells (Fig. 3B (panels i and ii)). Immunoelectron microscopy of the broken, agarose-embedded stably transfected N2A cells shows that HA-MC4R-GFP, labeled by anti-GFP antibodies, localized to coated pits at the plasma membrane (Fig. 3C (panels i and ii), arrows). No gold immunostaining was detected in parallel samples treated without primary antibodies (not shown). Post-embedding immunoelectron microscopy with anti-GFP (20 nm particle) and anti-clathrin antibodies (10 nm gold particles) showed co-localization of HA-MC4R-GFP and clathrin (Fig. 3C (panels iii–vi)). When N2A cells transiently expressing HA-MC4R-GFP were incubated with TMR-Tf, endocytosed Tf co-localized with a fraction of intracellular HA-MC4R-GFP (Fig. 3D). Moreover, when N2A cells transiently expressing HA-MC4R and exogenous TR were incubated at 37 °C with the anti-HA antibodies and TMR-Tf, a fraction of the internalized anti-HA-HA-MC4R complexes and Tf co-localized (Fig. 3D (panels iv–vi)). This shows that MC4R constitutive internalization to TR-containing endosomes is independent of the GFP tag. These experiments and those of Fig. 2 indicate that MC4R is constitutively internalized together with TR by clathrin-dependent endocytosis. A fraction of intracellular MC4R had a different distribution than TR (Fig. 3D, arrowheads) indicating that endocytosed MC4R localizes to other compartments in addition to those traversed by TR.

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dynamin1, inhibits TR internalization by blocking the formation of clathrin-coated vesicles (14). We reasoned that if constitutive MC4R internalization were also via clathrin-coated vesicles, then this process would be inhibited by expression of the dynamin1 mutant. In N2A cells transiently co-transfected with dynamin1 K44A and HA-MC4R-GFP, the rate of internalization of the receptor was inhibited by ~50% as compared with control cells (cells transfected with control plasmid, t½ = 6.63; cells transfected with dynamin1 K44A t½ = 13.62, respectively). In addition, the plateau was different with 22.82 and 39.14% of initial MC4R remaining at the cell surface in cells transfected with the control plasmid and dynamin1 K44A, respectively. Thus, in cells expressing the dynamin mutant, a fraction of HA-MC4R-GFP was not internalized and another fraction was internalized at a slower rate. Co-transfection with wild-type dynamin1 had no significant effect on the rate or extent of MC4R internalization. The experiment in Fig. 4C is consistent with the data in Fig. 3 indicating that constitutive MC4R internalization occurs by clathrin-dependent endocytosis.

If the amount of HA-MC4R at the cell surface remains constant at steady state, the receptor must be constitutively exocytosed to compensate for its continuous retrieval from the plasma membrane. If this were the case, an increasing population of receptors would be labeled during the incubation with the anti-HA antibody at 37 °C with the process reaching a plateau when all the intracellular receptors in equilibrium with the plasma membrane are labeled (Fig. 5A). To determine whether this is the case, cell surface HA-MC4R-GFP was labeled by the anti-HA-POD antibody at 4 °C. The incubation with the antibody was then continued at 37 °C, and the amount of cell surface and total HA-MC4R-GFP labeled at different time points was measured by incubation of unpermeabilized and permeabilized cells, respectively, with the POD substrate. The experiments were carried out with cycloheximide-treated cells to prevent labeling of HA-MC4R-GFP reaching the plasma membrane along the biosynthetic route. By using this approach, we found that HA-MC4R at the cell surface remained constant, whereas the total amount of receptor labeled by the antibody increased over time and reached the plateau after ~1 h (Fig. 5B). From this experiment, it is derived that in N2A cells MC4R undergoes constitutive exocytosis with a t½ of 13.37 min. These experiments further validate the conclusion that the population of intracellular receptor in equilibrium with the plasma membrane is similar in size to that at the cell surface. Immortalized hypothalamic GT1-7 neurons also exocytosed constitutively the receptor (Fig. 5C). Note that in these experiments the intracellular population of receptors externalized to the plasma membrane is not prebound to antibodies. Thus, MC4R traffics constitutively whether or not in a complex with antibodies.

The data presented above indicate that MC4R is internalized with TR by a clathrin-dependent mechanism. We have also shown that MC4R, like TR, is constitutively exocytosed. N2A cells express both SNAP-23 and SNAP-25, two soluble N-ethylmaleimide-sensitive factor attachment protein receptor components implicated in membrane docking and fusion (35). Both SNAP-23 and SNAP-25 function in constitutive recycling of endosomes (36–38). Thus, we expected that silencing of SNAP-23 expression, inactivation of SNAP-25, or both would
affect recycling of TR in neuronal cells. We also expected that if MC4R were exocytosed by the same route as TR, then reduced SNAP-23 and SNAP-25 activity would also inhibit MC4R traffic to the plasma membrane. We have derived from parental N2A cells the G14 cell line, which stably expresses BoNT/E light chain (35). BoNT/E is a bacterial protease that cleaves SNAP-25 and, to a lesser extent, SNAP-23 (39). In the G14 cells, virtually all of SNAP-25 was cleaved, whereas SNAP-23 remained intact (Fig. 6A). Transient transfection of N2A and G14 cells with siRNA targeted to silence SNAP-23 expression reduced the level of the protein by more than 70%. The expression level of other soluble N-ethylmaleimide-sensitive factor attachment protein receptor components implicated in vesicular traffic at the plasma membrane, such as syntaxin 1 and VAMP-2 (40), and of a cytoskeletal protein, actin, remained unchanged by SNAP-23 silencing. Thus, inhibition of SNAP-23 expression was specific. We monitored TR recycling by following loss of internalized POD-conjugated TF from the cells. The recycling rate of TR was identical in G14 cells with inactivated SNAP-25 and in N2A cells with silenced SNAP-23 expression (Fig. 6, B and C). In contrast, G14 cells with inactivated SNAP-25 and lowered SNAP-23 expression had ~2.5-fold reduced rate of transferrin recycling (t_{1/2} = 15.03 min), as compared with the control (t_{1/2} = 5.90 min), (Fig. 6D). These experiments show that both SNAP-25 and SNAP-23 function in TR recycling. Constitutive exocytosis of HA-MC4R-GFP was unaffected in the G14 cells with reduced SNAP-23 (Fig. 6E), suggesting that intracellular MC4R traffics to the plasma membrane by a different route than TR.

In the HEK 293 cells, exposure to α-MSH reduces the amount of MC4R at the plasma membrane (15, 16). Similarly, incubation of N2A cells with α-MSH reduced the amount of cell surface MC4R by ~40% (Fig. 7A). Because we find here that in neuronal cells MC4R is endocytosed in the absence of the agonist, we explored whether exposure to α-MSH decreased cell surface MC4R by enhancing the rate of receptor internalization. The rate of MC4R internalization was identical in cells treated in the absence and in the presence of α-MSH (Fig. 7B). It has been reported that MC4R has constitutive activity on which AGRP acts as an inverse agonist (41, 42). Incubation with AGRP did not change the amount of HA-MC4R-GFP at the cell surface or the rate of MC4R endocytosis (Fig. 7, C and D). Together, these experiments indicate that MC4R internalization is independent of receptor activity. Next, we determined whether reduced cell surface receptor in response to α-MSH is because of impaired traffic of the receptor back to the plasma membrane. To measure MC4R recycling defined as the receptor return to the cell surface after at least one round of internalization, we labeled

![Constitutive Traffic of MC4R](image_url)

**FIGURE 4.** Constitutive internalization of MC4R is rapid and dynamin-dependent. N2A cells stably expressing HA-MC4R-GFP were incubated with POD-conjugated anti-HA antibodies at 4 °C for 1 h, washed, and further incubated at 37 °C. A, cells were fixed under nonpermeabilizing conditions and incubated with the POD substrate to measure HA-MC4R-GFP at the cell surface. B, cells were fixed, permeabilized with detergent, and incubated with the POD substrate to measure total HA-MC4R-GFP. C, N2A cells were transiently co-transfected with HA-MC4R-GFP and either with empty pCB vector or with DynWT-pCB or DynK44A-pCB plasmids. Internalization rate of MC4R was measured as in A.

**FIGURE 5.** MC4R is constitutively exocytosed. A, schematic illustration of individual steps of the experiments shown in B and C. B and C, cycloheximide-treated N2A cells stably expressing HA-MC4R-GFP (B) and GT1-7 cells transiently expressing HA-MC4R-GFP (C) were incubated with POD-conjugated anti-HA antibodies at 4 °C. Cells were transferred to 37 °C in the continued presence of anti-HA-POD and cycloheximide. At different time points cells were fixed and washed, and the cell surface and total HA-MC4R-GFP labeled by the POD-conjugated anti HA-antibody were measured as in Fig. 4.
populations of MC4R that were endocytosed either in the absence or in the presence of α-MSH. N2A cells expressing HA-MC4R-GFP were first incubated at 37 °C in the absence or in the presence of the hormone with POD-labeled antibodies against HA to internalize the anti-HA/HA-MC4R-GFP complex. Cells were then treated with a low pH medium to strip the antibody from the population of receptors at the cell surface. Recycling of MC4R was followed by measuring, upon transfer of cells at 37 °C, the reappearance of POD-conjugated anti-HA/HA-MC4R-GFP at the cell surface (Fig. 7E). In the absence of α-MSH, MC4R cycled back to the plasma membrane with a t_{1/2} of 11.57 min. This is similar to the t_{1/2} of ~13 min measured when HA-MC4R-GFP exocytosis was followed in the absence of anti-HA antibody prebound to the receptor (Fig. 5B). It is concluded that HA-MC4R-GFP exocytoses with similar kinetics whether or not in a complex with the antibody against the HA tag. When MC4R was internalized in the presence of α-MSH, it recycled to the cell surface with a similar rate (t_{1/2} = 11.45 min) as that in the absence of the agonist (Fig. 7E). However, the percentage of internalized receptor that re-appeared at the plasma membrane was different with 41.7% and 26.8% of the internalized HA-MC4R-GFP recycling to the plasma membrane in cells treated without and with α-MSH, respectively. Thus, in N2A cells, 35% of the internalized MC4R pool that
Constitutive Traffic of MC4R

![Figure 8](image)

**FIGURE 8. Constitutive traffic of MC4R is important for signaling.** A, BFA reduces the amount of cell surface MC4R. N2A cells stably transfected with HA-MC4R-GFP pretreated with cycloheximide were incubated with BFA in the continuous presence of cycloheximide for 2 h. Cells were washed and fixed, and surface HA-MC4R-GFP was measured as in Fig. 1. Double asterisks, p < 0.01. B, cells were treated with BFA as in A, and the rate of MC4R exocytosis was measured as in Fig. 5B in the continuous presence of BFA. C, BFA-induced reduction of cell surface MC4R leads to a decrease in α-MSH induced cAMP formation. Cells were treated with BFA as in A. Cells were then stimulated with 100 nM α-MSH or with 10 μM forskolin, and cAMP production was measured as in Fig. 1A. Asterisk, p < 0.05.

Constitutive traffic of MC4R is important for signaling. In neuronal cells, the intracellular population of MC4R does not correspond to a pool of newly synthesized receptors because it persisted in cycloheximide-treated cells. Moreover, experiments where the antibody-feeding approach was combined with acid stripping of the antibody-MC4R complexes at the cell surface showed that it is the pool of internalized receptors that re-distributed rapidly to the plasma membrane. We estimated that at any given time, the size of the intracellular pool of MC4R that is in equilibrium with the plasma membrane is approximately the same as that at the cell surface. Binding to α-MSH induces both activation of the receptor and its de-sensitization (16). A possible functional significance of the MC4R recycling pathway is to maintain a pool of intracellular receptors that can continuously replenish those de-sensitized by exposure to the ligand at the cell surface. This is the case for another GPCR, the protease-activated receptor-1 where loss of the intracellular pool of protein leads to impaired re-sensitization after exposure to the ligand (21).

In unstimulated HEK 293 cells, MC4R is localized at the plasma membrane, and clathrin-dependent endocytosis of the receptor occurs upon exposure to α-MSH (15) and is dependent on agonist-induced phosphorylation of the receptor and its interaction with β-arrestin (16). In contrast to these observations, we find that MC4R is externalized constitutively. Because the level of cell surface receptor is constant, compensatory endocytosis must also occur constitutively, whether or not the receptor is in a complex with the antibody. Constitutive traffic of MC4R was rapid, with the entire pool of cell receptors exposed to the surface within a 1-h time interval. These observations are different from those reported by others in the embryonic kidney cells, where HA-MC4R-GFP was internalized only upon α-MSH stimulation (15, 16). We considered the possibility that high levels of expression of exogenous MC4R in the stably and transiently transfected N2A cells could lead to constitutive endocytosis because of receptor mis-targeting. In this respect, an immunoassay based on an antibody that recognizes the endogenous receptor and functional assays measuring α-MSH-dependent increase of cAMP suggest that exogenous MC4R in the stably transfected N2A clone represents only a fraction of the endogenous receptor. Transiently transfected N2A cells with more than 6-fold difference in exogenous MC4R expression level internalized the receptor with similar rates, suggesting that the process is independent of receptor density in the model under study.

DISCUSSION

In this study, we show that in specialized N2A and GT1-7 cells, ligand-free MC4R traffics constitutively. In support of this conclusion, various facts are provided. First, in the absence of α-MSH, it is found that the receptor has both a plasma membrane and an intracellular distribution. Second, a fraction of plasma membrane and intracellular MC4R co-localized with TR and MC4R was found in clathrin-coated pits and vesicles. Third, antibody-feeding experiments revealed that the receptor is constitutively internalized and exocytosed back to the plasma membrane. Constitutive HA-MC4R-GFP traffic might be a consequence of antibody binding to the tag. However, we consider this possibility unlikely. The assay to monitor MC4R exocytosis of Fig. 5 measures the appearance at the plasma membrane of receptors that are not pre-bound to antibodies. By this assay, we find that MC4R is externalized constitutively. Because the level of cell surface receptor is constant, compensatory endocytosis must also occur constitutively, whether or not the receptor is in a complex with the antibody. Constitutive traffic of MC4R would otherwise traffic back to the plasma membrane is blocked by exposure to α-MSH in the intracellular localization. In GT1-7 cells (Fig. 7F) MC4R recycled with a similar rate in the absence and in the presence of α-MSH (a t1/2 of 15.33 and 14.11 min, respectively). In the absence and in the presence of α-MSH, 43.6 and 29.1%, respectively, of internalized receptor re-appeared at the plasma membrane. Thus, similarly to N2A cells, exposure of immortalized hypothalamic neurons to the agonist blocks MC4R recycling by 33%, without changing the rate by which the process occurs. From the data in Fig. 7, it is concluded that the reduction of cell surface MC4R observed in response to α-MSH exposure is due an exocytotic block affecting a fraction of the internalized receptors and not to a reduced rate of receptor internalization.

Impaired constitutive recycling of MC4R might lead to reduced signaling by the receptor. To test this possibility, we determined whether BFA, a fungal metabolite that disrupts Golgi apparatus and endosome cell traffic (43), inhibited constitutive exocytosis of MC4R. Preincubation of N2A cells with BFA inhibited by ~50% the rate of MC4R exocytosis (t1/2 = 13.66 and 29.60 min in the absence and in the presence of BFA, respectively) and reduced by a similar extent the amount of MC4R at the cell surface (Fig. 8, A and B). Incubation with BFA had no effect on forskolin-induced cAMP generation indicating that adenylate cyclase activity is unaffected by the treatment (not shown). BFA reduced by ~30% the amount of cAMP generated in response to 15 min of exposure to α-MSH (Fig. 8C). These data suggest that impaired constitutive traffic of MC4R to the plasma membrane affected its ability to signal.
tions, we find that in N2A and GT1-7 cells, clathrin-dependent internalization of MC4R occurs constitutively. In addition, incubation of N2A cells with the agonist or with the inverse agonist AGRP did not change the rate of MC4R retrieval from the plasma membrane. MC4R, similarly to another GPCR, the \( \alpha_1 \)-adrenergic receptor, may undergo activity-independent and \( \beta \)-arrestin-dependent internalization (44, 45). Another possibility is that constitutive MC4R endocytosis occurs by a \( \beta \)-arrestin-independent mechanism. Such a process has already been described for some GPCR. For example, it has been shown that a viral GPCR, the chemokine receptor US28, is constitutively endocyctosed by a clathrin-dependent and \( \beta \)-arrestin-independent mechanism (33). In addition, endocytosis of the protease-activated receptor-1 is also dependent on a clathrin and dynamin-dependent process, but it is independent of \( \beta \)-arrestin (46).

The experiments described here show that in N2A and GT1-7 internalized MC4R is rapidly recycled and that reappearance of MC4R at the plasma membrane is strongly dependent on exposure to the agonist. This finding, together with the observation that the internalization rate of MC4R is unchanged by exposure to \( \alpha \)-MSH, suggests that agonist binding functions to reduce the amount of receptor expressed at the plasma membrane by affecting its traffic exclusively at the intracellular localization. This is a novel finding in the field of GPCR traffic, because even those receptors that are constitutively recycled, such as the thyrotropin receptor and the protease-activated receptor-1, maintain a distinct agonist-dependent internalization step (17, 20, 47, 48).

Here we find that a fraction of MC4R is internalized together with TR, but the intracellular pathways of TR and MC4R then diverge, because SNAP-25 inactivation and reduced SNAP-23 did not change the rate of MC4R exocytosis while impairing TR recycling. In addition, immunofluorescence experiments showed that a fraction of internalized MC4R does not co-localize with TR. A possible route of MC4R traffic to the plasma membrane is by traversing late endosomes and the trans-Golgi network. Along such route, \( \alpha \)-MSH-bound receptor could either traffic from late endosomes to lysosomes, thus heading the receptor for destruction, or be arrested at some point along the pathway until dissociation from the hormone and possibly from \( \beta \)-arrestin would make the receptor able to traffic back to the plasma membrane in a re-sensitized form. The experiments presented here show that both in neuroblastoma cells and immortalized hypothalamic neurons, a substantial fraction of MC4R internalized in the presence of \( \alpha \)-MSH traffics back to the plasma membrane. This is different from the HEK 293 cells, where MC4R exposed to the ligand did not recycle back to the plasma membrane and was targeted to lysosomes (15). The ability of MC4R internalized in the presence of \( \alpha \)-MSH to recycle back to the plasma membrane may be specific to neuronal cells and constitute another potential target to modulate receptor delivery at the cell surface.

In conclusion, we have demonstrated here that MC4R traffics constitutively and rapidly both in neuroblastoma cells and immortalized hypothalamic neurons. Although the exact nature of the neurons that control appetite and energy expenditure has not yet been established, our findings suggest that constitutive traffic of MC4R is a shared feature among neurons with different origins. So far, analysis of MC4R traffic has been limited to that along its biosynthetic route or to that induced by exposure to \( \alpha \)-MSH. The continuous cycling of MC4R in the N2A and GT1-7 cells suggests that defects along either the internalization or the externalization segment of the pathway may lead to changes in the number of receptors that reside at the plasma membrane and, ultimately, to the ability of the cell to respond to \( \alpha \)-MSH stimulation. In support of that, we find that exposure to BFA, a drug that impairs MC4R constitutive exocytosis, leads to a decreased pool of MC4R at the plasma membrane and to reduced production of cAMP upon exposure to \( \alpha \)-MSH. However, because BFA has multiple effects on membrane traffic, more work is necessary to determine whether inhibition of MC4R constitutive exocytosis and/or endocytosis affects receptor signaling. Given the centrality of MC4R function in the control of food uptake and energy homeostasis, it is of interest to determine whether onset of human obesity is linked to defects along the MC4R recycling pathway.

Acknowledgments—We are grateful to Dr. Richard I. Weiner (University of California San Francisco) for the gift of GT-7 cells; Dr. Timothy E. McGraw (Weill Medical College of Cornell University) for the gift of the plasmid pCDTR with human TR; Dr. Sandra Schmid (Scripps Research Institute) for the gift of Dyn WT-pCB and DynK44A-pCB with wild-type and mutated dynamin; Dr. Brian Storrie for helpful discussions and critically reading the manuscript; and Dr. Khaled Machaca and Dr. Grover Paul Miller for help with kinetic analysis of data.

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