Lipid stress inhibits endocytosis of melanocortin-4 receptor from modified clathrin-enriched sites and impairs receptor desensitization

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Melanocortin-4 receptor (MC4R) is a G-protein–coupled receptor expressed in the brain’s hypothalamus where it regulates energy homeostasis. MC4R agonists function to lower food intake and weight. In this respect, although obesity promotes hyperlipidemia and hypothalamic injury, MC4R agonists are nevertheless more effective to reduce food intake within hours of administration in overweight, rather than lean, mice. MC4R undergoes constitutive internalization and recycling to the plasma membrane with agonist binding inducing receptor retention along the intracellular route and, under prolonged exposure, desensitization. Here, we found that, in neuronal cells and lipid-stressed cells, lipid stress by exposure to elevated palmitate leaves unchanged the rate by which MC4R and transferrin receptor are constitutively excluded from the cell surface. However, lipid stress disrupted later steps of MC4R and transferrin receptor internalization to endosomes as well as traffic of agonist-occupied MC4R to lysosomes and MC4R desensitization. In the lipid-stressed cells, MC4R and clathrin were redistributed to the plasma membrane where they colocalized to sites that appeared by super-resolution microscopy to be modified and to have higher clathrin content than those of cells not exposed to elevated palmitate. The data suggest that lipid stress disrupts steps of endocytosis following MC4R localization to clathrin-coated sites and exclusion of the receptor from the extracellular medium. We conclude that increased effectiveness of MC4R agonists in obesity may be an unexpected outcome of neuronal injury with disrupted clathrin-dependent endocytosis and impaired receptor desensitization.

The melanocortin-4 receptor (MC4R) is a G-protein–coupled receptor (GPCR) abundantly expressed in the paraventricular nuclei of the hypothalamus and in the amygdala from where it controls several body functions including food intake and energy expenditure (1–4). MC4R is part of the melanocortin system where hormones, such as leptin and insulin secreted in the bloodstream by adipocytes and by the β-cells of the pancreas, respectively, cross the blood–brain barrier to bind to leptin and insulin receptors on the surface of pro-opiomelanocortin neurons localized in the arcuate nucleus of the hypothalamus. Leptin and insulin promote secretion of α-melanocyte-stimulating hormone (α-MSH) by neuropeptides that project to reach MC4R neurons in the paraventricular nucleus. In the paraventricular nucleus, released α-MSH interacts with MC4R to stabilize the receptor in an active conformation with Gs-mediated increase of intracellular cAMP and Gq-mediated increase of intracellular calcium (5, 6). Signaling by MC4R in response to α-MSH is thought to decrease food intake and to increase energy expenditure. Obesity, such as that induced by increased energy intake from hypercaloric diets with increased content of saturated fatty acid, induces hypothalamic injury with resistance to insulin and leptin, loss of α-MSH abundance, endoplasmic reticulum stress, and inflammation (7–12). A likely cause of hypothalamic injury in obesity is the increased levels of circulating non-esterified fatty acids (NEFAs), such as palmitic acid (9, 13). We have modeled neuronal injury by elevated palmitate by exposing neuroblastoma Neuro2A cells expressing endogenous and exogenous MC4R and immortalized hypothalamic neurons expressing endogenous MC4R to albumin-conjugated palmitate within the range of concentrations found in the blood of obese rodents and humans (14). In these cells, exposure to elevated palmitate induced loss of MC4R protein, detected by reduced abundance of the tagged MC4R reporter hemagglutinin (HA)-MC4R-GFP, and MC4R function, monitored by the decrease in the cAMP and AMP-activated protein kinase responses to acute stimulation by α-MSH (14). In contrast, it has also been reported that, in mice that are obese because of high-fat (HF) diet, the melanocortin system is instead over-responsive to MC4R agonists, such as the synthetic peptide melanotan II (MTII), within hours and days of agonist administration (10, 15). The finding that, in rodents fed

**The abbreviations used are:** MC4R, melanocortin-4 receptor; GPCR, G-protein–coupled receptor; α-MSH, α-melanocyte-stimulating hormone; MTII, melanotan II; Tf, transferrin; TFR, transferrin receptor; Tf-Rh, rhodamine-conjugated transferrin; Tf-biotin, biotin-conjugated transferrin; DIQ, diet-induced obesity; NEFAs, non-esterified fatty acids; HF, high-fat; ABTS, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); WGA, wheat germ agglutinin; ROI, region of interest; POD, peroxidases; RFP, red fluorescent protein; mRFP, monomeric red fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; MIP, maximum intensity projection; AUC, area under the curve.
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HF diet, there is over-responsiveness to MC4R agonists in the face of MC4R loss and general hypothalamic injury raised the question of whether some effects by lipid stress could actually promote MC4R function. We have reported that, in either the absence or presence of agonist exposure, MC4R cycles between the plasma membrane and endosomes by being continuously endocytosed by a mechanism that is dependent on cholesterol and clathrin (16, 17). Others and we have also found that, in the presence of agonist, there is increased MC4R retention in the endosomal/lysosomal compartment and receptor desensitization (17–20). In the present study, we found that lipid stress induced by exposure to elevated palmitate impairs clathrin-dependent endocytosis, redistributes MC4R to the plasma membrane, disrupts traffic of the receptor to endosomes/lysosomes, and impairs receptor desensitization.

Results

Exposure of Neuro2A_{HA-MC4R-GFP} Cells to elevated palmitate blunts MC4R desensitization.

A single intraperitoneal injection of MTII has more protracted effects to inhibit food intake in obese than in lean rodents within a few hours after delivery (10). To test whether there were differences by MTII treatment on weight loss, diet-induced obesity (DIO) male mice were treated for 15 weeks either with lean diet where 10% of calories are derived from fat or with high-fat diet where 60% of calories are derived from fat. The mice on a HF diet weight significantly more than the mice on the lean diet (Fig. 1A). Three hours before delivery of 150 nmol of MTII, food was removed from the cages and then returned to the cages immediately after agonist delivery (Fig. 1B). In the following 16 h, mice conditioned with the HF diet lost ~1% of their weight, whereas mice treated with the lean diet did not (Fig. 1C). Thus, obesity promotes, rather than inhibits, weight loss in response to MTII shortly after administration of the agonist. In contrast, we previously found that lipid stress, modeled in vitro by treating stably transfected Neuro2A_{HA-MC4R-GFP} cells expressing tagged MC4R and immortalized hypothalamic neurons with elevated palmitate, reduced the cAMP and AMP-activated protein kinase response to acute challenge by the natural MC4R agonist α-MSH (14). Consistent with that, in Neuro2A_{HA-MC4R-GFP} cells treated with albumin-conjugated palmitate (150–200 μM), the increase...
of intracellular cAMP signal induced by acute (15-min) exposure to MTII was significantly lower than that of cells not exposed to the elevated fatty acid (Fig. 1, D and F). Another component of the overall cAMP response by MC4R is receptor desensitization with decreased amplitude of the cAMP signal being generated in response to protracted agonist exposure (17, 18, 20, 21). When cells exposed for 18 h to 200 μM palmitate were treated with MTII for a protracted time (5 h; Fig. 1, D and F), the loss of the cAMP signal was less profound (~34%) than that of cells not exposed to the elevated fatty acid. This blunted decrease of the cAMP response by protracted agonist exposure took place also when cells were exposed to 200 μM palmitate for only 4 h and treated with α-MSH (Fig. 1, G and H). Together, the data indicate that MC4R exposure to elevated palmitate not only lowers the receptor acute cAMP response but also impairs MC4R desensitization. MC4R is constitutively internalized and recycled back to the plasma membrane, and desensitization of MC4R is thought to take place by retention of the receptor in the endosomal compartment and routing to lysosomes (17–19, 21). MC4R desensitization should then be dependent on lysosomal integrity, which is disrupted by cell exposure to the drug chloroquine (22). When Neuro2AHA-MC4R-GFP cells were treated with chloroquine, desensitization of MC4R was virtually abolished (Fig. 1I), thus suggesting that lysosome function is indeed essential for MC4R desensitization. These data suggest that exposure to elevated palmitate may impair internalization of MC4R and/or routing of the receptor to lysosomes.

Lipids stress changes Neuro2A cell shape and cell distribution of MC4R without altering the rate by which the receptor constitutively disappears from the cell surface

It has been reported that exposure to elevated palmitate induces cell stress with inhibition of cytokinesis (23). To visualize effects of lipid stress, Neuro2A cells were preconditioned with and without the addition of 150 and 200 μM palmitate. Wheat germ agglutinin (WGA) is a plant lectin that binds to glycoproteins or glycolipids containing sialic acid and N-acetyl-glucosamine residues at the plasma membrane and at the Golgi and trans-Golgi compartments (24). In Neuro2A cells treated without the added palmitate, Alexa Fluor 647-conjugated WGA faintly labeled the plasma membrane (Fig. 2A, arrows) and more intensely labeled intracellular organelles near the nucleus (arrowheads). Exposure of Neuro2A cells to 150 and 200 μM palmitate increased cell area by ~33 and ~56%, respectively (Fig. 2B). In addition, cells exposed to 150 and 200 μM palmitate were more frequently binucleated than control cells with an increased average area of DAPI-stained nuclei per cell (by 13 and 23%, respectively; Fig. 2, C and D). Thus, lipid stress changes Neuro2A cell shape and inhibits mitosis.

It has been found that resumption of clathrin-dependent endocytosis during late mitosis is required for cytokinesis (25). It is possible that, in the cells treated with elevated palmitate, defects in endocytosis may contribute to inhibit mitosis and affect other processes, such as constitutive internalization of MC4R (17). In Neuro2AHA-MC4R-GFP cells, HA-MC4R-GFP has the HA epitope attached to the N terminus of the protein and exposed to the extracellular medium and the GFP attached to the C terminus of the protein and exposed to the cytosol (Fig. 2E). To visualize possible effects of lipid stress on MC4R traffic and distribution, live Neuro2AHA-MC4R-GFP cells were incubated with the anti-HA antibody at 4 °C and then fixed. HA-MC4R-GFP at the cell surface was detected by secondary staining of non-permeabilized cells with Cy5-conjugated antibodies (pseudocolored in blue; merged image). A region of interest (ROI) was drawn outside of the plasma membrane, visualized by the staining with the anti-HA antibody (Cy5 fluorescence) (Fig. 2G). Treatment of Neuro2AHA-MC4R-GFP cells with 200 μM palmitate led to a 35% decrease of total GFP fluorescence within the ROI, indicating loss of HA-MC4R-GFP abundance, consistent with a previous report (14). Conversely, exposure to elevated palmitate increased the ratio Cy5 pixel intensity/GFP pixel intensity within the ROI by ~47%, indicating cell redistribution of HA-MC4R-GFP with an increased fraction of the total receptor residing at the cell surface.

Initial steps of constitutive internalization of MC4R have been monitored by measuring the disappearance of the HA epitope of HA-MC4R-GFP from the cell surface. To determine whether Neuro2AHA-MC4R-GFP cells exposed to elevated palmitate have an altered rate of receptor disappearance from the plasma membrane, cells were incubated with the anti-HA antibody at 4 °C to labeled the cohort of receptors at the cell surface and then transferred to 37 °C for different time intervals (17). The population of HA-MC4R-GFP with the HA epitope exposed to the cell medium (surface receptor) at the indicated time points was measured after cell fixation by using POD-conjugated anti-HA antibodies under non-permeabilizing conditions (Fig. 2H). The rate of receptor disappearance from the cell surface was similar (average t1/2 = ~9.9 min) regardless of whether Neuro2AHA-MC4R-GFP cells were exposed to the elevated palmitate. These experiments indicate that the rate by which the receptor constitutively disappears from the cell surface is not altered by lipid stress.

Exposure of Neuro2A cells to elevated palmitate inhibits constitutive endocytosis of MC4R to the intracellular compartment

To determine whether the cell redistribution of HA-MC4R-GFP in Neuro2AHA-MC4R-GFP cells exposed to elevated palmitate is paralleled by altered internalization of MC4R at steps later than that of receptor disappearance from the plasma membrane, Neuro2AHA-MC4R-GFP cells pretreated with and without elevated palmitate were briefly incubated at room temperature for 5 min with the anti-HA antibody to label HA-MC4R-GFP at the cell surface (Fig. 3A). Then the cells were transferred to 37 °C for the indicated time intervals, permeabilized, and treated with the Cy5-conjugated secondary antibody to visualize traffic of the anti-HA–HA-MC4R-GFP complex from the plasma membrane to the intracellular compartment (Fig. 3B). The process is monitored measuring the ratio fluorescence intensity of Cy5 in endosomes/fluorescence intensity of total GFP (Fig. 3C). In the Neuro2AHA-MC4R-GFP cells that were not exposed to elevated palmitate, the receptor labeled at the cell surface by the anti-HA antibody reached the intracellular location within 5 min (arrows). These data are consistent with other experiments where we have found similar fast internal-
The intracellular compartment in cells treated without prior exposures to low temperatures (4 °C). However, when Neuro2AHA-MC4R-GFP cells were preconditioned with 200 μM palmitate, the process of HA-MC4R-GFP endocytosis to the intracellular location was slower with HA-MC4R-GFP appearing at this location at the 15-min time point and a further increase of receptor abundance at the 60-min time point. Together, data in Figs. 2 and 3 indicate that, in lipid-stressed cells, MC4R at the cell surface is constitutively excluded from the extracellular medium rapidly like the receptor in the control cells not exposed to the elevated palmitate. However, in the lipid-stressed cells, the MC4R excluded from the cell surface appears to persist at the plasma membrane rather than reach the intracellular location.

It is possible that, in cells exposed to elevated palmitate, MC4R at the plasma membrane localizes to modified clathrin-coated vesicles that have impaired traffic to intracellular endosomes. To study possible changes of MC4R cell localization, we carried out cell fractionation experiments. The postnuclear supernatants of Neuro2AHA-MC4R-GFP cells treated with and without 200 μM palmitate were centrifuged to yield a P2 membrane pellet containing most of the plasma membrane marker Na+/K+-ATPase (Fig. 3, D and E). When P2 membranes derived from control cells not exposed to the elevated fatty acid

Figure 2. In Neuro2AHA-MC4R-GFP cells, exposure to elevated palmitate changes cell shape and cell distribution of MC4R without altering constitutive disappearance of the receptor from the cell surface. A, Neuro2A cells were treated with either no palmitate, 150 μM palmitate (palm), or 200 μM palmitate. Cells were stained with wheat germ agglutinin conjugated to Alexa Fluor 647 and with DAPI. B, total cell area (n cells > 100 per condition). C, areas of the nuclei (n cells > 100 per condition). D, average number of cells with two or more nuclei (n cells > 50 per condition). E, the upper drawing shows the HA and GFP tags of HA-MC4R-GFP, and the diagram shows the outline of the experiment shown in F. PM, plasma membrane. F, the HA-MC4R-GFP residing at the cell surface of Neuro2AHA-MC4R-GFP cells is labeled by the anti-HA antibody (Cy5; blue fluorescence), and cell HA-MC4R-GFP is visualized by the intrinsic GFP fluorescence (green fluorescence). G, GFP pixel intensity measures abundance of cell HA-MC4R-GFP (total receptor). The fraction of HA-MC4R-GFP localized at the cell surface was calculated as the ratio surface receptor pixel intensity (Cy5 fluorescence within ROIout)/total receptor pixel intensity (GFP fluorescence within ROIout). Cells were visualized with an Olympus Fluoview FV1000 confocal microscope. Each symbol corresponds to the value measured from a single cell. Cells are derived from three independent experiments. Scale bars, 10 μm. H, time course of HA-MC4R-GFP disappearance from the cell surface of Neuro2AHA-MC4R-GFP cells either with no palmitate addition or 200 μM palmitate. Values are normalized to the value at time t = 0 and are fitted using GraphPad Prism 6 software with a one-phase exponential decay equation. The experiment was done twice with similar results. Error bars represent S.D. Statistical significance is indicated as follows: ns, p > 0.05; *, p ≤ 0.05; **, p ≤ 0.01; ****, p ≤ 0.0001.
Figure 3. In Neuro2A-NC4R-GFP cells, exposure to elevated palmitate inhibits constitutive endocytosis of NC4R to the intracellular compartment. A, diagram of the experiment to measure internalization of tagged HA-NC4R-GFP in Neuro2A-NC4R-GFP cells. B, Neuro2A-NC4R-GFP cells incubated with rat anti-HA antibodies to monitor internalization of the population of NC4R at the cell surface were fixed, permeabilized, and stained with Cy5-conjugated anti-rat IgG. Arrows indicate HA-NC4R-GFP internalized to endosomes. Arrowheads indicate HA-NC4R-GFP localized at the cell surface. C, the ratio HA-NC4R-GFP in endosomes (fluorescence intensity of Cy5 in ROI in (white tracing in merged image)/total cell HA-NC4R-GFP (fluorescence intensity of HA-NC4R-GFP in ROI out (green tracing in merged image)) was quantified for each cell (n cells = 50 per condition). Scale bar, 10 μm. The experiment was done three times with similar results. D, diagram of Neuro2A-NC4R-GFP cell fractionation experiments to obtain P2 and P3 fractions. E, equal volumes (20 μl) of postnuclear supernatants (T), P2 pellet resuspended to 400 μl, and of P3 pellet resuspended to 200 μl derived from cells treated with either no palmitate addition or with 200 μM palmitate (palm) were analyzed by Western blotting using the indicated antibodies. F, the P2 pellets from cells treated with either no palmitate addition or with 200 μM palmate, obtained as outlined in D, were resuspended in homogenization buffer, loaded onto the sucrose gradient (30–50%, w/v), and analyzed by Western blotting with the indicated antibodies. Abundance of the HA-NC4R-GFP was quantified by band densitometry using ImageJ software. In the graphs, relative abundance of HA-NC4R-GFP in the gradient fractions is expressed as the percentage of the sum of HA-NC4R-GFP abundance in all fractions of the gradient. The relative abundance of Na⁺/K⁺-ATPase, clathrin, and TfR in each fraction was measured as described for that of HA-NC4R-GFP. The experiment was done twice with similar results. Error bars represent S.D. Statistical significance is indicated as follows: ns, p > 0.05; ****, p ≤ 0.0001.
were fractionated in parallel by sucrose density centrifugation, HA-MC4R-GFP migrated in the gradient as a peak that colocalized with that of Na\(^{+}/K^{+}\)-ATPase and with one of the clathrin peaks (fraction 5; Fig. 3F). When samples were derived from lipid-stressed cells, peaks of HA-MC4R-GFP comigrated with those of Na\(^{+}/K^{+}\)-ATPase and clathrin with the major peak of the receptor appearing at higher sucrose density than that of cells not treated with the elevated fatty acid in fraction 6. The data suggest that, in lipid-stressed and control cells, most of the HA-MC4R-GFP localizes to clathrin-coated vesicles of different composition, which would then change the sedimentation properties of plasma membrane vesicles across the gradient.

The internalization of transferrin (Tf) takes place by clathrin-dependent endocytosis of transferrin receptor (TfR) (26). It appears that MC4R and TfR are, at least in part, endocytosed together (17). In the sucrose gradient, TfR from the unboiled samples appeared with elevated apparent molecular weight consistent with the receptor existing as dimer (27) (Fig. 3F). The TfR derived from control cells migrated as major peak in fraction 2 in a low-density region of the sucrose gradient that also had a clathrin peak. Conversely, when samples were derived from cells exposed to the elevated palmitate, the TfR peak colocalizing with clathrin in fraction 2 did not appear, and two TfR peaks instead colocalized with those of HA-MC4R-GFP, Na\(^{+}/K^{+}\)-ATPase, and clathrin at higher density. The data indicate that lipid stress alters the cellular localization of TfR and suggest that, under this condition, TfR redistributes to the plasma membrane.

**Exposure of Neuro2A cells to elevated palmitate inhibits constitutive endocytosis of TfR to the intracellular compartment without altering the rate of receptor disappearance from the cell surface**

Our data indicate that constitutive internalization of MC4R to the endosomal compartment is altered in Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells exposed to elevated palmitate without changes in the rate by which the receptor disappears from the cell surface. To monitor TfR disappearance from the cell surface, Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells were incubated with biotin-conjugated Tf (Tf-biotin) at 4 °C to label the population of TfR residing at the cell surface. After transfer of the cells to 37 °C for different time intervals (Fig. 4A), the population of TfR remaining exposed to the cell surface was measured by treating the non-permeabilized cells with POD-conjugated streptavidin, which binds tightly to biotin. By this assay, the rate of receptor disappearance from the cell surface was similar (average \(t_{1/2}\) of \(-11.6\) min) regardless of whether Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells were exposed to the elevated palmitate. These experiments indicate that the rate by which the TfR receptor constitutively disappears from the cell surface is not altered by cell exposure to elevated palmitate like that of MC4R. We reasoned that lipid stress might instead impair the internalization of TfR from the plasma membrane to intracellular locations. To determine whether this is the case, Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells were exposed to Tf-biotin at 37 °C to allow the formation of TfR–Tf-biotin complexes. Then cells were washed once and then immediately and briefly exposed at room temperature to streptavidin-Alexa Fluor 647 to label the cohort of TfR–Tf-biotin complexes residing at the cell surface (Fig. 4D, arrows). When Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells not exposed to elevated palmitate were transferred to 37 °C, the TfR–Tf-biotin complexes labeled by streptavidin-Alexa Fluor 647 internalized from the plasma membrane (Fig. 4D, arrows) to the intracellular compartment (arrowheads) rapidly (\(t_{1/2}\) of \(-2.2\) min, \(R^2 = 0.63\)). In contrast, when Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells were exposed to the elevated palmitate, the streptavidin-Alexa Fluor 647 fluorescence remained for a longer time interval (15 min) at the plasma membrane (Fig. 4, D and E), indicating that the TfR–Tf-biotin complex persists at this location. When TfR–Tf-biotin complex appeared at the intracellular location at the 30-min time point, it had reduced abundance as compared with that of cells not exposed to the elevated palmitate with a decrease in the ratio endosomal Alexa Fluor 647 fluorescence/total cell Alexa Fluor 647 fluorescence (by \(-2.0\) fold, \(p < 0.0001\)).

When immortalized mHypoE-N42 hypothalamic neurons expressing endogenous MC4R (14, 21, 28) were exposed to rhodamine-conjugated transferrin (Tf-Rh) for 0.5 min at 37 °C, virtually all of the Tf-Rh was distributed at the cell surface above the layer of cortical actin at the cell margin (Fig. 4, F and G, arrows). When cells were kept at 37 °C for another 15 min in the continuous presence of Tf-Rh, the fluorescent protein not only labeled the plasma membrane more intensely but also appeared at the intracellular compartment (Fig. 4G, arrowheads) with an increase in the ratio pixel intensity of intracellular Tf-Rh/pixel intensity of total Tf-Rh (by \(-85\%\); Fig. 4, G and H), thus indicating Tf-Rh internalization. However, when mHypoE-N42 neurons were instead treated with 200 \(\mu\)M palmitate, Tf-Rh appeared to remain at the plasma membrane, and the ratio Tf-Rh/total Tf-Rh did not change. These observations using the mHypoE-N42 hypothalamic neurons together with those using Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells indicate that exposure to elevated palmitate has general effects to inhibit clathrin-dependent endocytosis both in the Neuro2A cells and in immortalized hypothalamic mHypoE-N42 cells.

In the cell fractionation experiment of Fig. 3F, both TfR and clathrin had altered distribution across the sucrose density gradient when samples were derived from lipid-stressed cells. To visualize clathrin distribution in Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells, unpermeabilized cells were stained with the anti-HA antibody to visualize the cell surface and then permeabilized and stained with the anti-clathrin antibody (Fig. 4I). In untreated Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells, clathrin immunostaining appeared both at the plasma membrane and at an intracellular location (Fig. 4I, arrow and arrowhead). This is consistent with clathrin-coated vesicles forming both at the plasma membrane and at the trans-Golgi compartments (29). Conversely, in Neuro2A\(_{\text{HA-MC4R-GFP}}\) cells treated with 150 and 200 \(\mu\)M palmitate, the pool of clathrin at the intracellular location appeared decreased with a reduction in the ratio intracellular clathrin pixel intensity/total clathrin pixel intensity (by \(-35\) and \(-55\%), respectively; Fig. 4K). Thus, lipid stress induces redistribution of cell clathrin with sequestration of the protein at the plasma membrane. At this location, clathrin fluorescence appeared clustered to individual spots in cells treated with and without elevated palmitate. Thus, at the plasma mem-
Figure 4. Lipid stress inhibits constitutive endocytosis of transferrin to the intracellular compartment in Neuro2A HA-MC4R-GFP and mHypoE-N42 hypothalamic neurons. A, diagram of the experiment to measure disappearance of Tf-biotin from the cell surface of Neuro2A HA-MC4R-GFP cells. B, constitutive internalization of Tf-biotin in Neuro2A HA-MC4R-GFP cells with no palmitate addition or treated with 200 μM palmitate (palm). Values are expressed as the percentage of that at the initial time point (t = 0) and are fitted using GraphPad Prism 6 software with a one-phase exponential decay equation. C, diagram of the experiment to measure internalization of Tf-biotin in Neuro2A HA-MC4R-GFP cells. D, Neuro-2AHA-MC4R-GFP cells were incubated with Tf-biotin to label the cell population of TfRs and then exposed for 2 min to streptavidin-Alexa Fluor 647 to monitor internalization of the Tf–TfR complex from the plasma membrane to the intracellular compartment. Arrows indicate streptavidin-Alexa Fluor 647 at the plasma membrane. Arrowheads indicate streptavidin-Alexa Fluor 647 internalized to endosomes. E, the ratio streptavidin-Alexa Fluor 647 in endosomes (fluorescence intensity of ROI∞ (white tracing))/total cell streptavidin-Alexa Fluor 647 (fluorescence intensity of ROIout (green tracing)) was quantified for each cell (n cells = 30 per condition). The experiment was done three times with similar results. F, diagram of the experiment shown in G. G, mHypoE-N42 hypothalamic neurons incubated with Tf-Rh and costained with Alexa Fluor 488 phalloidin. H, the ratio Tf-Rh in endosomes (fluorescence intensity of Tf-Rh in ROI∞ (blue tracing))/total cell Tf-Rh (fluorescence intensity of Tf-Rh in ROIout (magenta tracing)) was quantified for each cell (n cells = 60 per condition). All images were taken using an Olympus Fluoview FV1000 confocal microscope. I, diagram of the experiment shown in J. J and K, effects of Neuro-2AHA-MC4R-GFP cell exposure to elevated palmitate on clathrin distribution (n cells = 50 per condition). Scale bars, 10 μm. Each symbol in all panels except B corresponds to the value measured from a single cell. Cells are derived from three independent experiments. Error bars represent S.D. Statistical significance is indicated as follows: ns, p > 0.05; **, p ≤ 0.001; ****, p ≤ 0.0001.
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brane, clathrin distributes to clathrin-enriched sites regardless of cell exposure to lipid stress.

In cells treated with elevated palmitate, MC4R localizes to modified clathrin-coated sites

Data presented in this study indicate that MC4R and TfR disappear from the cell surface at the same rate in cells treated with and without elevated palmitate, and yet the two receptors have delayed traffic from the plasma membrane to the intracellular compartment. To determine whether MC4R indeed reaches clathrin-enriched sites in lipid-stressed cells, Neuro2AHA-MC4R-GFP cells treated with and without elevated palmitate were imaged by super-resolution microscopy. The focus was first set at a focal plane corresponding to the middle of the cell (Fig. 5A, middle) where, in palmitate-treated and untreated Neuro2AHAMC4R-GFP cells, HA-MC4R-GFP and clathrin fluorescence at the plasma membrane are clustered along a line corresponding to the cell margin. Then the focal plane was moved toward the cell surface (Fig. 5A, surface) where HA-MC4R-GFP and clathrin fluorescence appeared as scattered dots that colocalized to the same elevated extent in palmitate-treated and untreated cells as indicated by single-cell analysis using the Pearson’s coefficient (Fig. 5B). These data indicate that HA-MC4R-GFP indeed localizes to clathrin-coated pits at the cell surface regardless of lipid stress. Analysis of individual dots of GFP pixel intensity by the segment profile analysis tool (Fig. 5C) indicated that the amount of HA-MC4R-GFP in the dots was the same in cells treated with and without elevated palmitate (Fig. 5D). However, in the same dots, clathrin fluorescence intensity was more intense, by ~40%, when the Neuro2AHA-MC4R-GFP cells were exposed to the elevated palmitate (Fig. 5E). In addition, in cells treated with 200 μM palmitate, the average diameter of the dots as well as the median diameter of the dots appeared to be increased (by ~27 and 23%, respectively) as compared with those of control cells (Fig. 5F). Thus, in lipid-stressed cells, MC4R reaches clathrin-coated structures that are different from those of control cells because they have, for the same amount of receptor, an increased amount of clathrin and increased diameter. Dynamin functions to constrict the neck of clathrin-coated vesicles and catalyzes membrane fission (30). Here we asked whether HA-MC4R-GFP at the plasma membrane colocalizes with dynamin in cells exposed to the elevated palmitate. Lipid stress actually increased colocalization of HA-MC4R-GFP with dynamin by ~39% (Fig. 5, G and H). Thus, MC4R may reach clathrin-coated sites where the receptor is excluded from the cell surface by the dynamin collar. These data are in agreement with cell fractionation experiments suggesting that HA-MC4R-GFP residing at the plasma membrane localizes with modified clathrin-coated sites.

Exposure of Neuro2A cells to elevated palmitate inhibits MC4R endocytosis to Rab5 endosomes and lysosomes

After clathrin shedding, internalized vesicles undergo Rab5-dependent homotypic fusion to form early endosomes, which then migrate toward the cell center to generate late endosomes (31). We have found that, in Neuro2A cells, MC4R traffic appears disrupted at steps where the receptor is endocytosed to the intracellular location (Fig. 3, A–C). Here we asked whether MC4R indeed traverses the Rab5A endosomes and whether such traffic is impaired in cells exposed to lipid stress. To this end, live Neuro2AHA-MC4R-GFP cells transiently expressing mRFP-Rab5 (32) were pulse-labeled for 1 min at 37 °C with the anti-HA antibody to label the population of HA-MC4R-GFP receptors at the cell surface and then either immediately fixed or transferred to 37 °C for 15 min to allow internalization of the anti-HA–HA-MC4R-GFP complex (Fig. 6A). Cells were then permeabilized and stained with Cy5-conjugated secondary antibodies to detect the cohorts of anti-HA-labeled receptors at the plasma membrane and at the intracellular location. By super-resolution microscopy, in control cells that were fixed immediately after the pulse with the anti-HA antibody, the anti-HA-coupled HA-MC4R-GFP appeared as spots of blue Cy5 fluorescence at the cell margins (Fig. 6B, arrowheads) and mRFP-Rab5 appeared concentrated intracellularly. After the 15-min chase at 37 °C, the Cy5-labeled HA-MC4R-GFP disappeared from the cell perimeter (indicated by a gray line) to distribute inside the cell with significantly increased colocalization with mRFP-Rab5 fluorescence (the region of Fig. 6B at the arrow is magnified in the inset; white color indicates colocalization). In contrast, in cells pretreated with 150 and 200 μM palmitate, after the 15-min chase at 37 °C, the Cy5-labeled anti-HA spots remained at the cell perimeter without increased colocalization with mRFP-Rab5 (Fig. 6C). Thus, the data indicate that, in cells exposed to lipid stress, traffic of HA-MC4R-GFP from the plasma membrane to the Rab5 endosomes is impaired.

When exposed to agonists, MC4R is routed to lysosomes in a process thought to contribute to MC4R desensitization (17–19). To determine whether lipid stress affects routing of agonist-exposed MC4R to lysosomes, Lamp1–RFP (33) was transiently expressed in Neuro2AHA-MC4R-GFP cells (Fig. 6, D and E). In Neuro2AHA-MC4R-GFP cells not exposed to elevated palmitate, after 1 h of continuous incubation with both MTII and anti-HA, the internalized HA–HA-MC4R-GFP complex colocalized with Lamp1–RFP to a greater extent than that in cells exposed to anti-HA for only 1 min (Fig. 6E, colocalization areas appear as white). Conversely, there was no significant increase of colocalization of the HA–HA-MC4R-GFP complex and Lamp1–RFP in cells pretreated with elevated palmitate. The data indicate that lipid stress impairs endocytosis of agonist-exposed MC4R to lysosomes, thus blunting MC4R desensitization.

Discussion

A cause of hypothalamic injury in obesity is the increased levels of circulating saturated NEFAs, such as palmitic acid (9, 13). A key finding of this work is that a feature of neuronal injury by cell exposure to elevated saturated fatty acids is inhibition of clathrin-dependent endocytosis with impaired internalization of MC4R and TfR to endosomes. In this respect, it appears that, although lipid stress does not change the rate by which MC4R and TfR are excluded from the cell surface, it impairs later steps in receptor endocytosis, such as entry into endosomes and lysosomes. In general, disruption of essential components of clathrin-coated vesicles, such as epsin and
Figure 5. In Neuro2AHA-MC4R-GFP cells exposed to elevated palmitate, HA-MC4R-GFP reaches modified clathrin-enriched sites at the plasma membrane. A, Neuro2AHA-MC4R-GFP cells were treated with and without palmitate (palm) as indicated and then immunostained for clathrin. For each field, the distribution of HA-MC4R-GFP and clathrin is visualized in middle of the cell (middle; scale bar, 10 μm) and at the cell surface (surface; scale bar, 10 μm; scale bar in the inset, 3.5 μm). B, colocalization of HA-MC4R-GFP (GFP fluorescence) and clathrin (Cy3 fluorescence) in clathrin-enriched sites from Neuro2AHA-MC4R-GFP cells imaged at the surface was monitored by Pearson’s correlation coefficient (n cells = 30 per condition). C, fluorescence intensity profiles of clathrin-enriched sites measured along a line drawn across individual dots of Cy3 (clathrin) and GFP (HA-MC4R-GFP) fluorescence. D–F, graphs show AUC values derived from the fluorescence intensity profiles of GFP (HA-MC4R-GFP; D) and Cy3 (clathrin; E) and the major diameter of the Cy3 fluorescence at the clathrin-enriched sites (dot diameter; F). Each symbol corresponds to one fluorescence dot (n dots > 100; derived from eight different Neuro2AHA-MC4R-GFP cells per condition). G, Neuro2AHA-MC4R-GFP cells were treated with and without palmitate as indicated and then immunostained for dynamin. The distribution of HA-MC4R-GFP and dynamin is visualized at the cell surface (surface; scale bar, 3.5 μm). H, colocalization of HA-MC4R-GFP (GFP fluorescence) and dynamin (Cy5 fluorescence) from Neuro2AHA-MC4R-GFP cells was monitored by Pearson’s correlation coefficient (cells treated without palmitate addition, n = 13; cells treated with 200 μM palmitate, n = 17). Data are derived from three independent experiments. Error bars represent S.D. Statistical significance is indicated as follows: ns, p > 0.05; **, p < 0.01; ****, p = 0.0001.
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dynamin, or cholesterol depletion has been found to change the shape and abundance of clathrin-coated regions (34–38). Indeed, analysis by super-resolution microscopy carried out here indicates that, in neuronal cells exposed to elevated palmitate, MC4R colocalizes with clathrin and dynamin, indicating that the receptor resides in clathrin-coated pits. However, such clathrin-enriched sites appear modified, as compared with those of control cells not exposed to the elevated fatty acid, because of their increased clathrin content and increased size. In addition, cell fractionation experiments suggest that MC4R,
TfR, and clathrin-containing plasma membrane vesicles of cells exposed to elevated palmitate have increased density as compared with those of cells treated without excess fatty acid. This finding is consistent with the conclusion that, in cells exposed to lipid stress, both MC4R and TfR localize at the plasma membrane to modified clathrin-coated pits that have dynamin assembled at their neck and where accessibility of the receptor to the extracellular medium is precluded (model of Fig. 6G).

In rodents fed HF diet, in the face of hypothalamic injury and resistance to leptin and insulin, there is over-response to MC4R agonists, monitored both within hours after agonist administration and upon a continuous agonist treatment lasting several days (Fig. 1) (10, 15). It has been proposed that the hyper-responsiveness to MC4R agonists in the HF diet-treated mice is due to increased abundance of MC4R. In this respect, it has been found that HF diet-treated mice have increased levels of MC4R mRNA (10, 39). However, hypothalamic binding of iodinated α-MSH appears to be decreased, rather than increased, in the hypothalamus of rats exposed to HF diet (40). Unfortunately, a direct measure of MC4R abundance in the hypothalamus has not yet been carried out due to a lack of sensitive antibodies against the receptor. In the Neuro2AHA-MC4R-GFP cells expressing low levels of the exogenous reporter HA-MC4R-GFP and exposed to an elevated concentration of albumin-conjugated palmitate to mimic lipid stress, abundance of the receptor, detected either by Western blot analysis (14) or by direct fluorescence microscopy (Fig. 2), is decreased. Moreover, lipid stress leads to loss of cAMP signal in response to acute exposure to MC4R agonists both in the Neuro2AHA-MC4R-GFP cells and in immortalized hypothalamic neurons (Fig. 1 and Ref. 14). Therefore, lipid stress either in the hypothalamus or modeled in cultured cells appears to reduce, rather than to increase, MC4R abundance. Conversely, others and we have found that desensitization of MC4R is another important component of the overall response of MC4R to agonists (18, 21). Here we found that, when Neuro2AHA-MC4R-GFP cells are exposed to lipid stress, MC4R desensitization is blunted, and internalization of HA-MC4R-GFP to endosomes as well as receptor routing to lysosomes is impaired. In respect to receptor desensitization, it has been proposed that arrestin binding blocks signaling by GPCR (41). However, more recently it has also been reported that GPCRs can exist in complexes that include both arrestin and G-protein and that such complexes can emanate sustained signaling (42). MC4R, like other GPCRs, undergoes constitutive internalization and recycling to the plasma membrane with agonist binding inducing receptor retention along the intracellular route by a process mediated, at least in part, by arrestin (17, 43). Whether lipid stress impairs MC4R desensitization by affecting receptor binding to arrestin in addition to inhibiting receptor routing to lysosomes remains to be established. In conclusion, the data presented here indicate that injury by lipid stress to alter MC4R traffic to lysosomes and desensitization, rather than effects on receptor abundance, may contribute to the increased response of MC4R to agonists in obesity.

We have previously found that lowering cell clathrin abundance, although inhibiting internalization of MC4R (16), also leads to loss of MC4R’s intrinsic ability to signal through Gs to induce increased intracellular cAMP. In contrast, in cells exposed to elevated palmitate, despite the impaired MC4R internalization reported here, the intrinsic ability of MC4R to signal is preserved (14). Together, these observations suggest that MC4R residency to clathrin sites, rather than receptor internalization and recycling, is essential to maintain the function of MC4R at the plasma membrane. Such possibility is consistent with the recent finding that clathrin-coated vesicles may serve as hubs to poise at least some GPCRs, such as the β-adrenergic receptor, to a second wave of cAMP signaling emanating from endosomes (44). In conclusion, the work presented here indicates that lipid stress by elevated NEFAs can modulate signaling by GPCRs by affecting receptor traffic and highlights the importance of monitoring the response to GPCR agonists in cells with altered metabolic state.

**Experimental procedures**

**Materials**

Lipofectamine 3000 was purchased from Life Technologies. Rat monoclonal anti-HA antibody (3F10 clone), rat monoclonal anti-HA-peroxidase high-affinity antibody 3F10 (12 013 819 001), streptavidin-POD conjugate (11089153001), ABTS buffer solution (11 112 597 001), and ABTS (10 102 964 001) were purchased from Roche Applied Sciences. Rh-Tf (T2872), DAPI nucleic acid stain (D1306), wheat germ agglutinin-Alexa Fluor 647 conjugate, Alexa Fluor 488 phallolidin, and transferrin from human serum bovin-XX conjugate (T-23363) were purchased from Molecular Probes. α-MSH, poly-l-lysine, 3-isobutyl-1-methylxanthine (IBMX), chloroquine, fatty acid-free bovine serum albumin (A7511-10G), sodium palmitate (P9767-5G), and EGTA (E-8145) were from Sigma-Aldrich. MITII was obtained from Tocris. The direct cAMP enzyme immunoassay kit was from Enzo Life Sciences, Inc. (ADI-901-066). Cy5-conjugated anti-rat IgG, Cy3-conjugated anti-rabbit IgG, peroxi-

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**Figure 6. Exposure of Neuro2AHA-MC4R-GFP cells to elevated palmitate inhibits endocytosis of HA-MC4R-GFP into early endosomes and lysosomes.** A, diagram of the experiment shown in B, B, Neuro2AHA-MC4R-GFP Cells were transfected with mRFP-Rab5 plasmid and treated with and without elevated palmitate (palm) as in Fig. 2. Live cells treated with rat anti-HA antibodies at 37 °C for 1 min were either fixed (0 min) or transferred to 37 °C for an additional 15 min to allow internalization of the anti-HA–HA-MC4R-GFP complex, fixed, and stained by secondary staining of permeabilized cells. Arrowheads indicate the anti-HA–HA-MC4R-GFP complexes (blue fluorescence) at the cell margins (gray tracing). The arrow indicates internalized HA–HA-MC4R-GFP complexes. Insets show internalized HA–HA-MC4R-GFP complexes and mRFP-Rab5 at 3-fold higher magnification. C, colocalization of the HA–HA-MC4R-GFP complex with mRFP-Rab5 expressed as Pearson’s coefficient (n cells ≥5 per condition). D, diagram of the experiment shown in E, E, arrowheads indicate the anti-HA–HA-MC4R-GFP complexes (blue fluorescence) at the cell margins. Arrows indicate internalized HA–HA-MC4R-GFP complex in the proximity of and/or colocalizing with Lamp1-RFP (white color). The lowest panels show Lamp1-RFP and internalized HA–HA-MC4R-GFP complexes at 3-fold higher magnification. F, colocalization of internalized HA–HA-MC4R-GFP complex and Lamp1-RFP is quantified as in C (n cells ≥4 per condition). G, proposed model of effects of lipid stress on MC4R and TfR traffic. PM, plasma membrane; E, endosome; Lys, lysosome. Data are derived from three independent experiments. Error bars represent S.D. Statistical significance is indicated as follows: ns, p > 0.05; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
dase-conjugated monoclonal mouse anti-rabbit IgG (211-032-171), and peroxidase-conjugated goat anti-mouse IgG (115-035-174) were purchased from Jackson ImmunoResearch Laboratories. Formaldehyde (16%) was from Ted Pella, Inc. G418 (30-234-CR) and Dulbecco’s modified Eagle’s medium (DMEM; 10-013-CV) were purchased from Corning Cellgro. The mHypoE-N42 hypothalamic neurons (N42 cells) were obtained from Cedarlane Laboratories. The mRFP-Rab5 plasmid was a gift from Ari Helenius (Addgene plasmid 14437), and Lamp1-RFP was a gift from Walther Mothes (Addgene plasmid 1817). Anti-clathrin heavy chain antibody rabbit (ab21679), mouse monoclonal anti-α1 Na+/K+ -ATPase (ab7671), and rabbit polyclonal anti-transferrin receptor antibody (ab84036) were purchased from Abcam. The lean 10% fat diet (product D12450B) and the 60% fat diet (product D12492) were purchased from Research Diets, Inc. Sucrose (BP220-1), EDTA (BP120-500), and Pierce™ protease inhibitor minitables (product A32955) were purchased from Thermo Fisher Scientific. Mouse monoclonal dynamin antibody (Hudy2) was purchased from Upstate Biotechnology Inc. (05-330). The ball-bearing cell homogenizer was obtained from Isobiotec Inc., Heidelberg, Germany. The Eppendorf MiniSpin (F-45-12-11) centrifuge was from Eppendorf Inc. The Beckman Optima (TLX) ultracentrifuge, Beckman centrifuge tubes (343778), fixed angle rotor (TLA 100.2), and swing bucket rotor (TLS-55) were purchase from Beckman Coulter Life Sciences.

**Animals**

The DIO C57BL/6J male mice purchased from The Jackson Laboratory were housed in a temperature-controlled environment with a 12-h light, 12-h dark cycle (lights on at 6 a.m. and lights off at 6 p.m.) and given ad libitum access to food and water. Starting at 6 weeks of age for the following 15 weeks, mice were conditioned with a lean diet containing 10% of calories derived from fat and with a HF diet with 60% calories derived from fat. On the day of the experiment, mice were fasted from 9:30 a.m. to 12:30 p.m. and then injected intraperitoneally with either saline or MTII in saline (150 nmol). Weight loss was measured the following morning at 9:30 a.m. by using an animal weighing scale. All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences.

**Preparation of bovine serum albumin (BSA)-conjugated palmitate**

The 100 mM palmitate stock solution was prepared in 0.1 M NaOH and kept at −20 °C. To prepare a solution containing BSA-conjugated palmitate, the 100 mM palmitate stock solution was heated to 72 °C in a water bath to obtain a clear solution. Fatty acid-free BSA was prepared as a 5% solution and then sterile filtered. The 100 mM palmitate stock was used to make a 5 mM solution of palmitate coupled to albumin by adding 50 μl of the 100 mM palmitate heated to 72 °C directly to 950 μl of 5% BSA heated to 37 °C. The 5 mM palmitate stock solution was diluted directly into complete medium to treat cells for 18 h. Control cells were treated in parallel with 5% BSA.

**Cell culture**

Neuro-2A_HA-MC4R-GFP cells and mHypoE-N42 hypothalamic neurons were cultured as previously described (14, 17).

**Desensitization assay**

Neuro2A cells, cultured as described previously (17), were plated onto 24-well plates ~24 h before the experiment. Cells were washed with either no palmitate, 150 μM palmitate, or 200 μM palmitate for 18 at 37 °C. Cells were washed three times with 250 μl of serum-free DMEM and incubated in 500 μl of serum-free DMEM for 1 h at 37 °C. Cells were then treated for 5 h with DMEM with 0 nm MTII (non-prechallenged samples) or 200 nm MTII in DMEM (prechallenged samples). Cells were then washed three times in 250 μl of DMEM. Both prechallenged and non-prechallenged samples were then treated with 500 μl of DMEM containing 500 μM IBMX for 10 min and then with 500 μl of DMEM with IBMX and either no MTII or 200 nm MTII for 15 min. Cells that were not pre-exposed to MTII were used as controls to measure cAMP generation in response to acute exposure to MTII. The cAMP concentrations were then measured using the Enzo Life Sciences complete ELISA kit and analyzed using GraphPad Prism 6 software using a nonlinear regression curve to calculate the concentration of cAMP in the samples. In some desensitization experiments, chloroquine (20 μM) was added for a total time interval of 16 h.

**Time course of constitutive HA-MC4R-GFP disappearance from cell surface**

The assay was carried out as described previously (17). Briefly, Neuro2A_HA-MC4R-GFP cells were placed in 24-well plates 24 h before the assay was performed. Cells were washed with DMEM at room temperature, incubated for 1 h at 37 °C in DMEM, transferred on ice, and incubated with DMEM containing POD-conjugated anti-HA antibody (1:500) at 4 °C for 1 h to label HA-MC4R-GFP at the cell surface. Cells were washed with cold DMEM and either kept on ice (time 0) or transferred to 37 °C for 5, 10, 15, 30, or 60 min. Cells were then fixed with 1 × PBS containing 4% formaldehyde at 4 °C for 10 min on ice and washed with PBS, and POD activity was measured as described above to measure disappearance of the cell incubation medium at 405 nm using an Epoch microplate spectrophotometer (Bio-Tek Instruments, Inc.).

**Time course of constitutive disappearance of biotin-conjugated transferrin from cell surface**

Neuro-2A_HA-MC4R-GFP cells were incubated with biotin-conjugated transferrin (25 μg/ml) at 4 °C for 1 h to label the cell surface transferrin receptors. Cells were washed and either kept on ice or transferred to 37 °C. Cells were then fixed with 1 × PBS containing 4% formaldehyde at 4 °C for 10 min on ice, washed with PBS, and POD activity was measured as described above to measure disappearance of HA-MC4R-GFP from the cell surface.
Cell fractionation

Neuro-2A_{HA-MC4R-GFP} cells were scraped in homogenization medium (250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.4) containing protease inhibitors. Cells were homogenized using an Isobiotec Inc. ball-bearing homogenizer with a 16-μm clearance. Next, the cell homogenate was centrifuged at 600 × g for 10 min at 4 °C to remove nuclei and unbroken cells using an Eppendorf MiniSpin centrifuge. The supernatant was then centrifuged at 5,000 × g with the same centrifuge, and the pellet (P2) was resuspended in 400 μl of homogenization medium. The supernatant was collected and centrifuged at 88,600 × g for 1 h at 4 °C in a fixed angle rotor (TLA 100.2) using a Beckman Optima™ TLX ultracentrifuge, and the pellet (P3) was resuspended in 200 μl of homogenization medium. The P2 fraction was placed on top of a 30–50% (w/v) discontinuous sucrose gradient. The gradient was then centrifuged with the same centrifuge in a swinging bucket rotor (TLS-55) at 165,000 × g for 2 h. Samples were collected as 150-μl fractions from the top and diluted with sample buffer before analysis by Western blotting.

DAPI and WGA staining of Neuro2A cells

Neuro2A cells plated on coverslips were treated with and without palmitate and fixed in 1× PBS containing 4% formaldehyde for 10 min on ice and an additional 20 min at room temperature. Cells were washed three times in PBS and incubated in a solution containing PBS, 0.1 μg/ml ovalbumin, and 0.2% Triton X-100. Cells were stained with wheat germ agglutinin conjugated to Alexa Fluor 647 (5 μg/ml) for 10 min at room temperature, washed three times in 1× PBS, and incubated with DAPI at a concentration of 14.3 μM for 15 min to stain nuclei. Cells were visualized using an Olympus Fluoview FV1000 confocal microscope. The cell area and the area of the nuclei were quantified by drawing with ImageJ software an ROI immediately outside the cell margin to measure the total cell area and another ROI immediately outside the blue DAPI staining to measure the area of the nuclei. The population of binucleated cells was quantified after exporting images using the FV10-ASW 4.2 viewer as tiff files and then by counting the number of cells with one or more nuclei, respectively, using the Plug-ins tab, Analyze tab, and Cell Counter tab of ImageJ software.

Total and surface HA-MC4R-GFP

Live Neuro-2A_{HA-MC4R-GFP} cells were incubated with the rat anti-HA antibody (1:500) for 2 h at 4 °C and then either kept at 4 °C or transferred to 37 °C for the indicated time period prior to fixation. The HA-MC4R-GFP residing at the cell surface was visualized by secondary staining with anti-rat Cy5-conjugated antibodies in non-permeabilized cells. Total cell HA-MC4R-GFP was quantified by measuring the GFP fluorescence intensity in an ROI drawn outside of the cell perimeter. To monitor the fraction of total receptor residing at the cell surface, the ratio HA-MC4R-GFP at the cell surface (Cy5 fluorescence in ROI_{out})/total receptor (GFP fluorescence in ROI_{out}) was quantified for each cell.

Internalization of HA-MC4R-GFP to intracellular compartment in Neuro-2A_{HA-MC4R-GFP} Cells

Cells were washed at room temperature and incubated with the anti-HA antibody (1:500) in DMEM for 5 min at room temperature. Cells were washed three times in DMEM at room temperature and then either transferred to 4 °C (time 0) or to 37 °C for the indicated time points (times 5, 10, 15, 45, and 60 min) prior to fixation. To visualize the internalized HA-MC4R-GFP over time, the cells were permeabilized in a solution containing PBS, 0.1 μg/ml ovalbumin, and 0.2% Triton X-100 and incubated with anti-rat secondary antibodies conjugated to Cy5. Total cell HA-MC4R-GFP was quantified by measuring the GFP fluorescence intensity in an ROI drawn outside the cell perimeter (ROI_{out}). To monitor the fraction of internalized receptor residing at the intracellular location, the Cy5 fluorescence intensity was measured in another ROI outlining the intracellular HA-MC4R-GFP (ROI_{in}). The ratio intracellular receptor pixel intensity (Cy5 fluorescence in ROI_{in})/total receptor pixel intensity (GFP fluorescence in ROI_{out}) was quantified for each cell per condition. Internalization of HA-MC4R-GFP over time was measured as the change in the ratio intracellular HA-MC4R-GFP (Cy5 fluorescence)/total HA-MC4R-GFP (GFP fluorescence).

Internalization of transferrin to intracellular compartment in Neuro-2A_{HA-MC4R-GFP} Cells

Cells were washed with DMEM at room temperature and incubated with biotin-conjugated transferrin (25 μg/ml) for 15 min at 37 °C. The medium was aspirated, and the cells were labeled with Alexa Fluor 647-conjugated streptavidin (10 μg/ml) for 2 min at room temperature, then washed once at room temperature, and transferred either to 4 °C (time 0) or to 37 °C for the indicated time points (times 5, 10, 15, and 30 min) to allow endocytosis to take place. Then cells were washed again three times with cold DMEM and fixed. Total cell transferrin was quantified by measuring the ratio Alexa Fluor 647-conjugated streptavidin bound to biotin-conjugated transferrin in an ROI drawn outside the cell perimeter (ROI_{out}) visualized by HA-MC4R-GFP at the plasma membrane. To monitor the fraction of internalized transferrin, another ROI was drawn immediately below the plasma membrane (ROI_{in}). The ratio intracellular transferrin pixel intensity (Alexa Fluor 647 fluorescence in ROI_{in})/total transferrin pixel intensity (Alexa Fluor 647 fluorescence in ROI_{out}) was quantified for each cell. Internalization of transferrin over time was measured as the change in the ratio intracellular transferrin (Cy5 fluorescence in ROI_{in})/total transferrin (Cy5 fluorescence in ROI_{out}).

Internalization of Tf-Rh in mHypoE-N42 hypothalamic neurons

The mHypoE-N42 hypothalamic neurons (N-42 cells) were exposed to Tf-Rh at 37 °C for 0.5 and 15 min, respectively. Cells were washed three times in 37 °C DMEM and immediately fixed, permeabilized, and stained for 30 min with Alexa Fluor 488 phalloidin at a concentration of 165 nM to visualize F-actin at the cell cortex. Images were taken with an Olympus Fluoview FV1000 confocal microscope. For each cell, two ROIs were drawn with one immediately below the cortical actin staining
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(ROI_{in}) and another outside of the cortical actin staining at the cell perimeter (ROI_{out}). The ratio Tf-Rh in endosomes (fluorescence intensity of Tf-Rh within ROI_{in})/total cell Tf-Rh (fluorescence intensity of Tf-Rh in ROI_{out}) was quantified for each cell.

Super-resolution microscopy: image acquisition

Neuro2A_{HA-MC4R-GFP} cells were fixed, permeabilized, and stained with rabbit anti-clathrin antibody and with secondary anti-rabbit Cy3-conjugated antibody. Images were taken using an ELYRA super-resolution Zeiss microscope. Distribution of clathrin and HA-MC4R-GFP was visualized from a set of six z-stack images. The range of the combined six z-stack slices was 0.50 μm, and the z-distance (interval) between the slices was 0.101 μm. To ensure that, within the same experiment, all images are taken with the same settings between conditions, a reference image from the same experiment was opened, and the Reuse tab was selected to acquire new images. The Channel Alignment tab was used to align the Cy3 and GFP channels and to create Structured Illumination_Channel Alignment CZI files with the z-stacks of six images.

Colocalization of HA-MC4R-GFP and clathrin

Colocalization was quantified by opening, with ZEN Blue software, the Structured Illumination_Channel Alignment CZI file obtained as described above; then, under the Colocalization tab, selecting the Costes tab (an automatic threshold setting); and then selecting Colocalization Tools and the Freehand (Bezier) icon to draw an ROI around the cell perimeter. For the selected ROI, the Pearson's coefficient for each of the six z-stack images was displayed by using the Table tab and the average Pearson's coefficient was then calculated.

Fluorescence intensity profile of Cy3 (clathrin) and GFP (HA-MC4R-GFP) and Cy3 fluorescence dot diameter

In ZEN Black software, from the Structured Illumination_Channel Alignment CZI files, the six z-stacks images were constructed together in a single image by creating a maximum intensity projection (MIP) image as follows. Under the Processing tab, the MIP tab was selected, then the Structured Illumination_Channel Alignment CZI file was selected, and Apply was selected. Files were then exported as MIP images. To measure the fluorescence intensity profiles of Cy3 (clathrin) and GFP (HA-MC4R-GFP), MIP images were opened using ZEN Blue software, and then the Profile tab was selected followed by the Dimensions tab to enlarge the image (by 160%). Then, under the Profile Definition tab, the Arrow icon was selected to draw an arrow across an individual spot of fluorescence. A chart appeared with the fluorescence intensity profiles, namely distance expressed in nm (x values) and the corresponding fluorescence intensity values of Cy3 and GFP (y values). These data were copied into Prism 6.0 software to generate graphs with the fluorescence intensity profiles of Cy3 (clathrin) and GFP (HA-MC4R-GFP). By using the area under the curve (AUC) tool, the integrated fluorescence intensity of Cy3 (clathrin) and GFP (HA-MC4R-GFP) was calculated for each dot. From the AUC, the longer diameter of the clathrin fluorescence dot was then calculated by subtracting the distance in nm between the last x and the first x of the peak.

Colocalization of Rab5 and HA-MC4R-GFP

Neuro2A cells were transiently transfected with 1 μg of mRFP-Rab5 plasmid (Addgene 14437) in 6-cm dishes. The following day the transfected cells were split onto coverslips and, once confluent, treated with and without palmitate. Cell surface receptors were then labeled with rat anti-HA antibodies at 37 °C for 1 min, washed, and either immediately fixed or transferred to 37 °C for 15 min to allow internalization of HA-MC4R-GFP coupled to anti-HA antibodies and then fixed. Cells were permeabilized and stained with secondary Cy5-conjugated antibody against rat IgG. Images were taken using an ELYRA super-resolution Zeiss microscope, and colocalization of Rab5 and HA-MC4R-GFP was visualized by setting the confocal plane to the middle of the cell and by creating a Structured Illumination_Channel Alignment CZI file as described above. Colocalization of HA-MC4R-GFP with mRFP-Rab5 was quantified by calculating the Pearson's correlation coefficient.

Colocalization of Lamp1-RFP and HA-MC4R-GFP

Neuro2A cells were transiently transfected with 1.5 μg of Lamp1-RFP plasmid (Addgene 1817) as described above. Colocalization of Lamp1-RFP and HA-MC4R-GFP was quantified by the Pearson's correlation coefficient.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software. Statistical significance was calculated by two-tailed unpaired t test on two groups or two-way analysis of variance when more than two groups were being compared with p < 0.05 considered statistically significant. Data are expressed as mean ± S.D.

Author contributions—K. A. C., B. M. M., N. S. K., and S. R. conducted the experiments. G. B. designed the experiments. K. A. C., B. M. M., S. R., and G. B. wrote the paper.

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