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Statin-Induced Chronic Cholesterol Depletion Switches GPCR Endocytosis and Trafficking: Insights from the Serotonin$_{1A}$ Receptor

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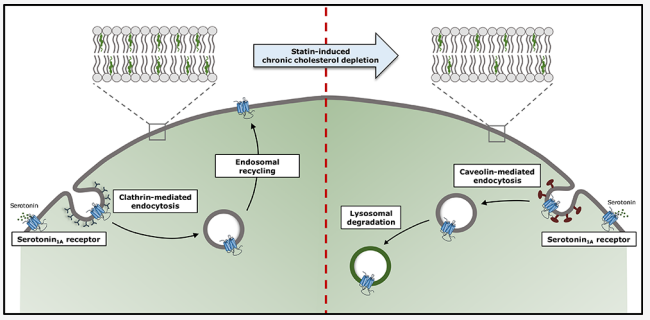
ABSTRACT: Endocytosis is a key regulatory mechanism adopted by G protein-coupled receptors (GPCRs) to modulate downstream signaling responses within a stringent spatiotemporal regime. Although the role of membrane lipids has been extensively studied in the context of the function, organization, and dynamics of GPCRs, their role in receptor endocytosis remains largely unexplored. Cholesterol, the predominant sterol in higher eukaryotes, plays a crucial role in maintaining the structure and organization of cell membranes and is involved in essential cellular processes in health and disease. The serotonin$_{1A}$ receptor is a representative GPCR involved in neuronal development and in neuropsychiatric disorders such as anxiety and depression. We recently combined quantitative flow cytometric and confocal microscopic approaches to demonstrate that the serotonin$_{1A}$ receptor undergoes clathrin-mediated endocytosis upon agonist stimulation and subsequently traffics along the endosomal recycling pathway. In this work, we show that statin-induced chronic cholesterol depletion switches the endocytic pathway of the serotonin$_{1A}$ receptor from clathrin- to caveolin-mediated endocytosis. Interestingly, under these conditions, a significant proportion of endocytosed receptors is rerouted toward lysosomal degradation. To the best of our knowledge, these results constitute one of the first comprehensive reports on the role of membrane cholesterol in GPCR endocytosis and trafficking. These results are significant in our overall understanding of the modulatory effects of membrane lipids on GPCR endocytosis and trafficking and could provide novel insight in developing therapeutic interventions against neuropsychiatric disorders such as depression.



KEYWORDS: *G protein-coupled receptors, serotonin$_{1A}$ receptor, endocytosis, intracellular trafficking, cholesterol, statin*

INTRODUCTION

G protein-coupled receptors (GPCRs) are plasma membrane associated signaling hubs that orchestrate a multitude of cellular functions upon binding to a wide variety of extracellular cues ranging from neurotransmitters to photons.[1−5] The emergence of GPCRs as major drug targets across all clinical areas is indicative of their relevance in a diverse array of pathophysiological conditions.[6−9] Although the intracellular signaling cascades mediated by GPCRs are complex, these downstream responses are maintained within stringently regulated physiological regimes. Endocytosis is a key regulatory mechanism employed by GPCRs to exercise spatiotemporal control over receptor-mediated signaling events.[10−12] Endocytosis enables spatial decoupling of the receptor from its extracellular ligand pool, which is accessible exclusively to receptors localized in the plasma membrane. This is achieved by internalization of the receptor into intracellular vesicular compartments called endosomes. Endocytosis was primarily regarded as a mechanism of receptor desensitization.[10] However, emerging research has revealed several noncanonical features of receptor endocytosis. It has been suggested that endocytosis could facilitate receptor-mediated signaling from intracellular locations distinct from the plasma membrane.[13−15]

The process of endocytosis involves a complex, yet coordinated interplay between a number of membrane-associated and cytoplasmic proteins, and membrane lipids, that contribute to recruitment of cargo, generation of local membrane curvature, and budding of cargo-laden vesicles from the plasma membrane.[16,17] In this context, several intracellular components of the endocytic machinery have been identified, yet our understanding of the role of membrane lipids in the process of endocytosis is rather limited.

GPCRs are intimately associated with their immediate membrane microenvironment owing to their seven transmembrane domain architechture and highly dynamic extramembranous loops that could interact with membrane lipids.[4,18] Cholesterol is an important lipid in membranes of

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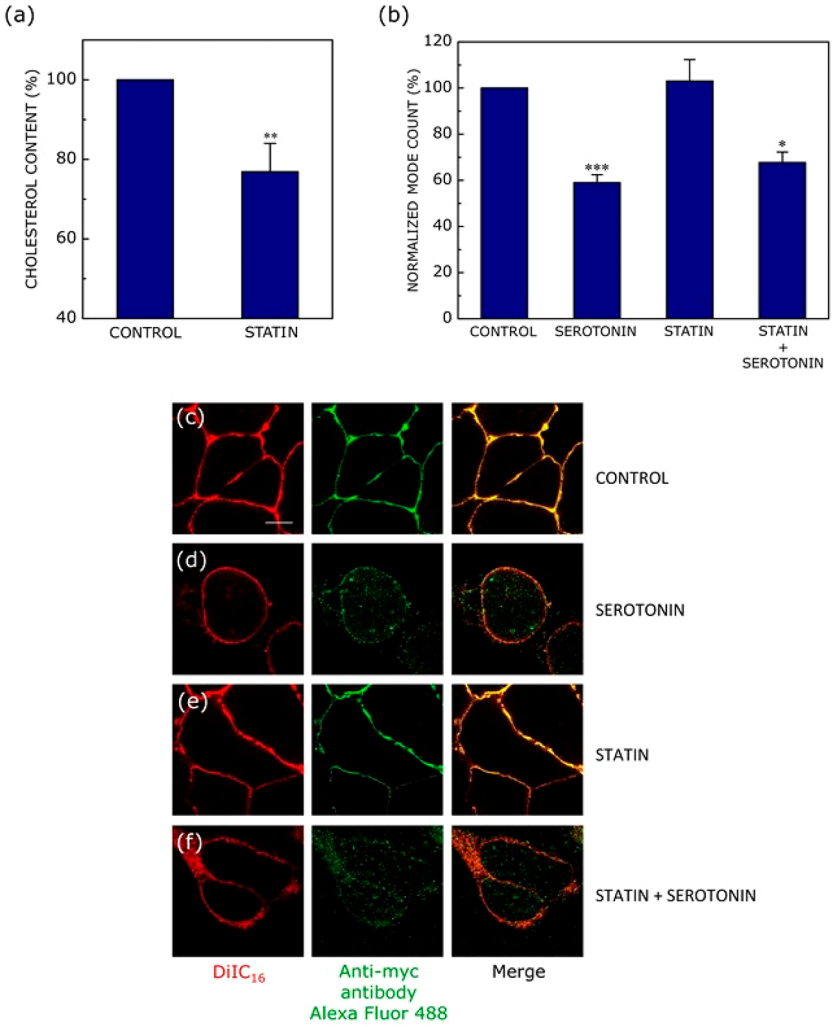

Figure 1. Statin-induced chronic cholesterol depletion does not inhibit serotonin$_{1A}$ receptor internalization. HEK-5-HT$_{1A}$R cells were treated with 5 μM lovastatin for 24 h prior to incubation with 10 μM serotonin for 60 min. (a) Cholesterol content in HEK-5-HT$_{1A}$R cells under control and statin-treated conditions. Values are normalized to cholesterol content in control (untreated) cells. Data represent means ± SE of at least four independent experiments (** corresponds to significant ($p < 0.01$) difference in cholesterol content in statin-treated cells relative to control cells). (b) Quantitative flow cytometric estimates of plasma membrane receptor population upon statin treatment. Values are normalized to mode count associated with control cells. Data represent means ± SE of at least four independent experiments (*** and * correspond to significant ($p < 0.001$ and $p < 0.05$) difference in mode count associated with cells incubated with serotonin and cells incubated with serotonin upon statin-treatment relative to control cells, respectively). (c–f) Representative confocal microscopic images of myc-tagged serotonin$_{1A}$ receptors labeled with anti-myc antibody Alexa Fluor 488 conjugate (green) and plasma membranes labeled with DiIC$_{16}$ (red) under control and statin-treated conditions (in the presence and absence of serotonin). The scale bar represents 10 μm. See Methods for other details.

higher eukaryotes and accounts for ∼30−50% of the total plasma membrane lipid content.[19,20] The unique structural attributes of cholesterol and its ability to nonrandomly organize into distinct membrane domains contribute to the functional role of cholesterol in signal transduction, trafficking, and pathogenesis.[21−26] Importantly, membrane cholesterol has been shown to modulate key functions of several GPCRs such as ligand binding, G-protein coupling, and downstream signaling.[27−34] However, the role of membrane cholesterol in GPCR endocytosis and intracellular trafficking remains largely unexplored.

The serotonin$_{1A}$ receptor is one of the most comprehensively studied members of the GPCR superfamily and has been implicated in neuronal development and neuropsychiatric disorders such as anxiety, depression, schizophrenia, and Parkinson's disease.[35−40] As a consequence, the serotonin$_{1A}$ receptor has emerged as a key molecular player in the development of antidepressant therapeutics such as selective serotonin reuptake inhibitors (SSRIs) that modulate the homeostasis of the serotonergic system as their mode of action.[41,42] Interestingly, endocytosis of the serotonin$_{1A}$ receptor has been shown to induce receptor desensitization which acts as the mechanism of action of SSRIs such as fluoxetine.[43,44] In this context, we have recently characterized the molecular mechanism underlying the endocytosis and intracellular trafficking of the human serotonin$_{1A}$ receptor utilizing quantitative flow cytometric and confocal microscopic approaches.[45] Our results showed that the serotonin$_{1A}$ receptor undergoes agonist-induced internalization via clathrin-mediated endocytosis and subsequently recycles back to the plasma membrane along the endosomal recycling pathway.

Extensive work from our laboratory has comprehensively demonstrated the role of membrane cholesterol in the organization, dynamics, function, and oligomerization of the

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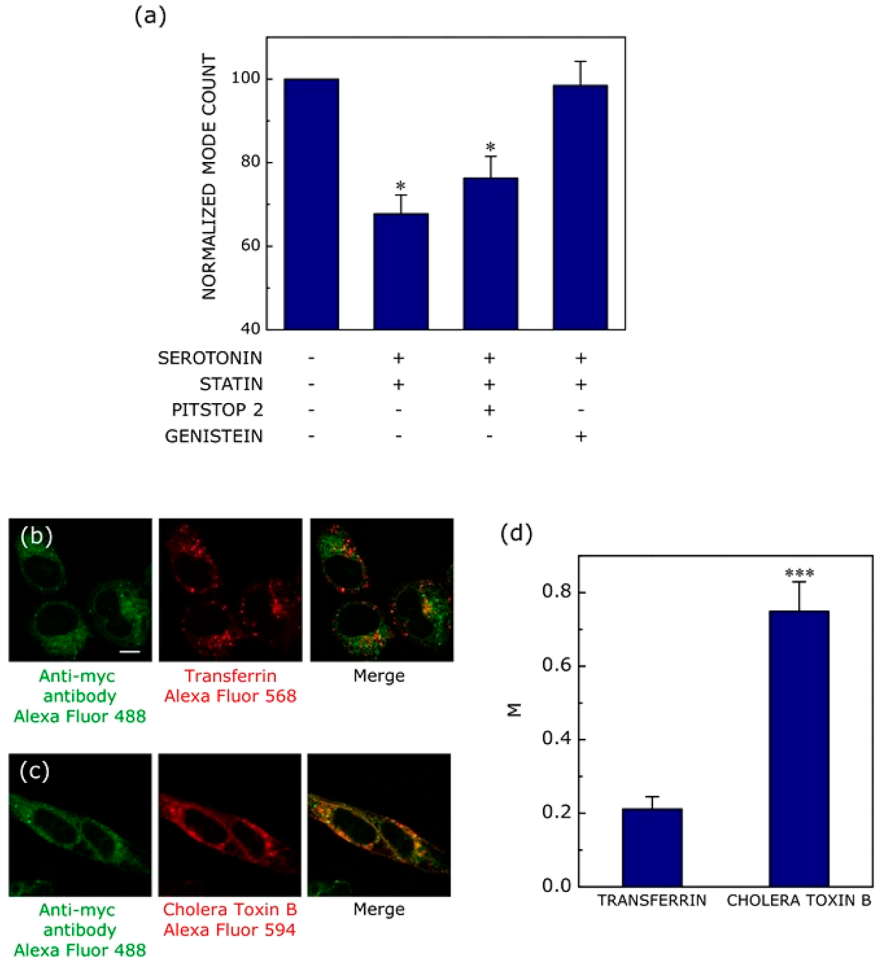

Figure 2. Statin-induced switch in the mechanism of serotonin$_{1A}$ receptor internalization. (a) Statin-treated cells were incubated with 10 μM serotonin for 60 min subsequent to treatment with specific inhibitors of clathrin-mediated endocytosis (pitstop 2) or caveolin-mediated endocytosis (genistein). The panel shows quantitative flow cytometric estimates of plasma membrane receptor population under these conditions. Values are normalized to mode count associated with control cells. Data represent means ± SE of at least four independent experiments (* correspond to significant ($p < 0.05$) difference in mode count associated with cells incubated with serotonin and serotonin in the presence of pitstop 2 under statin-treated conditions, relative to control cells). Representative confocal microscopic images of statin-treated HEK-5-HT$_{1A}$R cells labeled with anti-myc antibody Alexa Fluor 488 conjugate (green) and (b) transferrin Alexa Fluor 568 conjugated, or (c) cholera toxin B Alexa Fluor 594 conjugated (red). The scale bar represents 10 μm. (d) The extent of colocalization between serotonin$_{1A}$ receptor and transferrin (or cholera toxin B) under statin-treated condition quantitatively estimated using Manders' colocalization coefficient (M). Data represent means ± SE of at least 9 independent measurements (*** corresponds to significant ($p < 0.001$) difference in Manders' colocalization coefficient (M) associated with colocalization of the serotonin$_{1A}$ receptor with cholera toxin B relative to transferrin under statin-treated conditions). See Methods for other details.

serotonin$_{1A}$ receptor utilizing a combination of experimental and computational approaches.[46−55] In the present study, we have explored the role of cholesterol in the endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor utilizing a physiologically relevant chronic method of cholesterol depletion by statin. Statins constitute one of the best selling drugs in clinical history that act as competitive inhibitors of HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the cholesterol biosynthetic pathway.[56−59] Our results show that chronic cholesterol depletion by statin induces a switch in the endocytic pathway of the serotonin$_{1A}$ receptor from clathrin- to caveolin-mediated endocytosis. Importantly, we observed that metabolic replenishment of cholesterol restores the pathway of internalization to clathrin-mediated endocytosis. We further observed that upon chronic cholesterol depletion, a significant fraction of the internalized receptors is routed toward lysosomal degradation. To the best of our knowledge, these results constitute one of the first reports on the role of membrane cholesterol in GPCR endocytosis and intracellular trafficking. Our results assume significance in the overall understanding of the modulatory effects of membrane lipids on GPCR endocytosis and intracellular trafficking.

RESULTS

Statin-Induced Chronic Cholesterol Depletion Does Not Affect the Extent of Serotonin$_{1A}$ Receptor Endocytosis. We have recently explored the endocytosis and intracellular trafficking pathway of the serotonin$_{1A}$ receptor utilizing quantitative flow cytometric and confocal microscopic approaches.[45] For this purpose, we stably expressed the functional human serotonin$_{1A}$ receptor with a myc-tag at its N-terminus in HEK-293 (HEK-5-HT$_{1A}$R) cells.[45] Flow cytometry allows statistically robust quantitative analysis of

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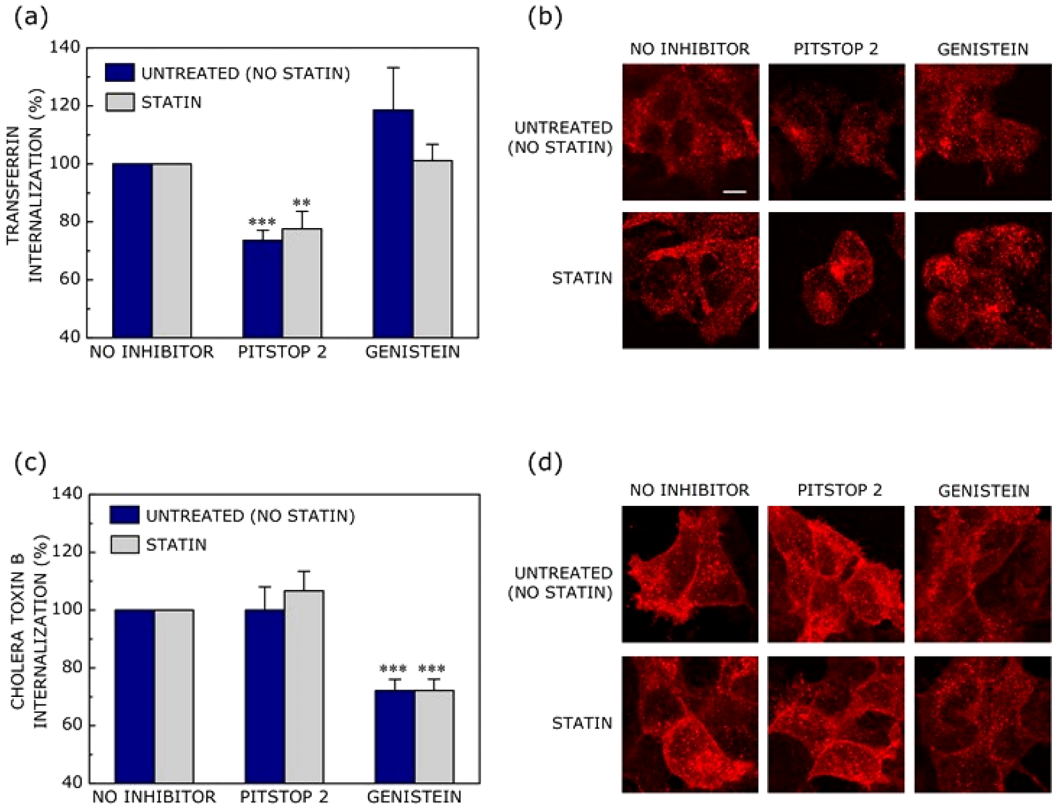

Figure 3. Chronic cholesterol depletion induced switch in the mechanism of endocytosis is specific to the serotonin$_{1A}$ receptor. The effect of statin-induced chronic cholesterol depletion on the mechanism of internalization of known markers of clathrin- and caveolin-mediated endocytosis was monitored by measuring the internalization of transferrin and cholera toxin B, respectively. (a) Extent of internalization of transferrin quantified as the number of transferrin positive puncta inside cells per unit area, normalized to control cells (no statin treatment) (blue) and statin-treated (gray) conditions. Data represent means ± SE of at least 6 independent measurements (*** and ** correspond to significant ($p < 0.001$ and $p < 0.01$) difference in the internalization of transferrin into HEK-5-HT$_{1A}$R cells treated with pitstop 2 relative to untreated cells, under control and statin-treated conditions, respectively). (b) Representative confocal microscopic images of the endocytosis of transferrin Alexa Fluor 568 conjugate under various conditions. (c) Extent of internalization of cholera toxin B quantified as the number of cholera toxin B positive puncta inside cells per unit area, normalized to control cells (no statin treatment) (blue) and statin-treated (gray) conditions. Data represent means ± SE of at least 5 independent measurements (*** corresponds to significant ($p < 0.001$) difference in the internalization of cholera toxin B into HEK-5-HT$_{1A}$R cells treated with genistein relative to untreated cells, under control and statin-treated conditions). (d) Representative confocal microscopic images of the endocytosis of cholera toxin B Alexa Fluor 594 conjugate under various conditions. The scale bar represents 10 μm. See Methods for other details.

fluorescence readouts from a population of cells. In our flow cytometric assay to quantify endocytosis of the serotonin$_{1A}$ receptor, we exclusively label the plasma membrane receptor population with anti-myc antibody Alexa Fluor 488 conjugate. Reduction in the plasma membrane receptor pool as a consequence of agonist-induced endocytosis is monitored as reduction in cell counts associated with the modal channel (mode count) on a flow cytometric histogram (see ref 45 and Methods for more details).

In order to understand the role of membrane cholesterol in the endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor, we depleted cholesterol from HEK-5-HT$_{1A}$R cells in a chronic fashion using lovastatin. Unlike acute methods of cholesterol depletion such as treatment with methyl-β-cyclodextrin (MβCD), which are associated with limitations due to a relatively short duration of treatment, chronic methods offer a more physiologically relevant approach to deplete cellular cholesterol.[60−62] In this context, statins (best selling cholesterol lowering drugs) offer a physiological approach to modulate cellular cholesterol levels in a chronic manner. Statins act by competitive inhibition of HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the cellular cholesterol biosynthetic pathway.[56,63]

Figure 1a shows that treatment of HEK-5-HT$_{1A}$R cells with 5 μM lovastatin for 24 h resulted in reduction in cellular cholesterol levels by ∼23%. Control experiments showed that cell viability is not compromised upon treatment with lovastatin (see Figure S1). As shown by us previously, the serotonin$_{1A}$ receptor undergoes endocytosis upon incubation with its native ligand serotonin.[45] Figure 1b shows that the extent of internalization of the serotonin$_{1A}$ receptor does not exhibit any significant difference (relative to cells not treated with lovastatin) upon chronic depletion of cholesterol, with ∼32% reduction in membrane receptor population when incubated with 10 μM serotonin for 60 min. Importantly, treatment with statin alone does not influence the plasma membrane receptor population (see Figure 1b). Figure 1c−f shows confocal microscopic images of HEK-5-HT$_{1A}$R cells with serotonin$_{1A}$ receptors labeled with anti-myc antibody Alexa Fluor 488 conjugated and the plasma membrane labeled with DiIC$_{16}$ under untreated and statin-treated conditions. As shown in Figure 1c,e, a predominant plasma membrane localization of the serotonin$_{1A}$ receptor was observed in control

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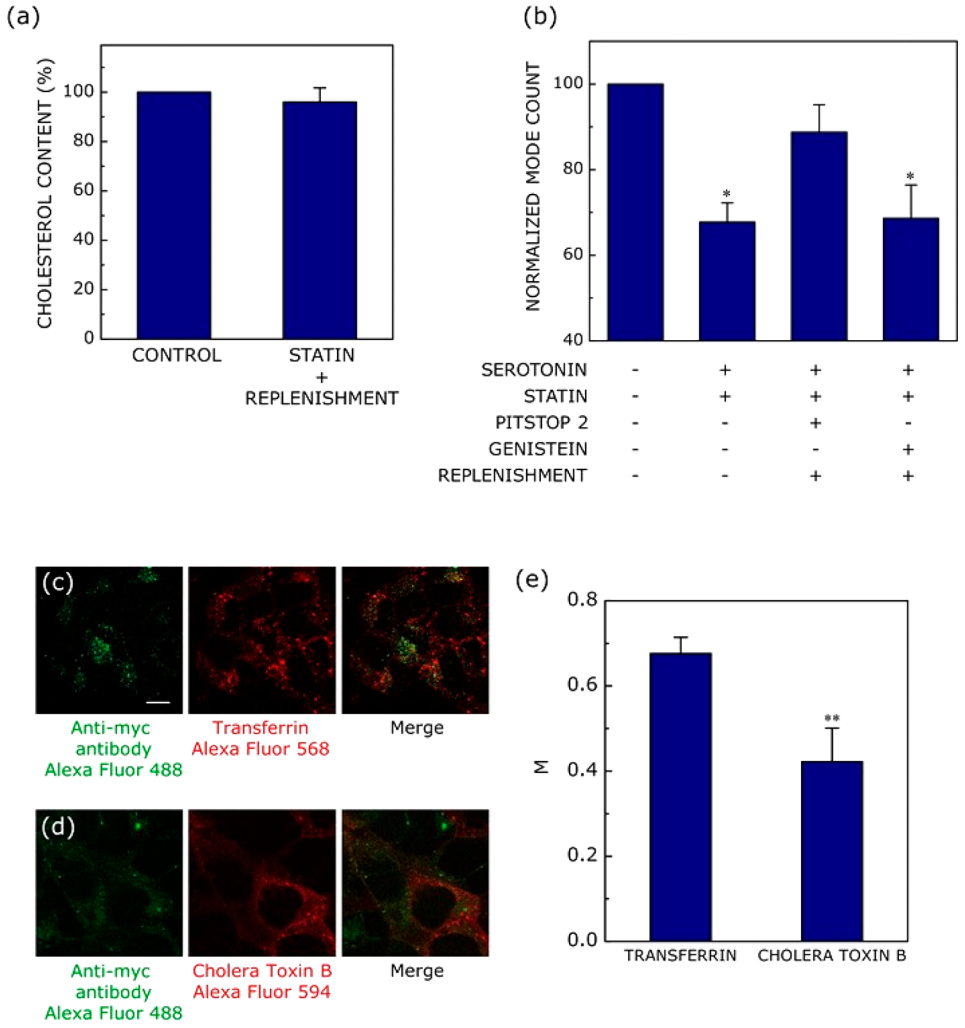

Figure 4. Chronic cholesterol depletion induced switch in the mechanism of serotonin$_{1A}$ receptor endocytosis is reversible in nature. Cholesterol was metabolically replenished in statin-treated HEK-5-HT$_{1A}$R cells by incubating cells with medium containing 20% serum. (a) Cholesterol content in HEK-5-HT$_{1A}$R cells under control and cholesterol-replenished (subsequent to statin treatment) conditions. Values are normalized to cholesterol content in control (untreated) cells. Data represent means ± SE of at least four independent experiments. (b) Cholesterol-replenished cells were incubated with 10 μM serotonin for 60 min subsequent to treatment with specific inhibitors of clathrin-mediated endocytosis (pitstop 2) or caveolin-mediated endocytosis (genistein). The panel shows quantitative flow cytometric estimates of plasma membrane receptor population under these conditions. Values are normalized to mode count associated with control cells. Data represent means ± SE of at least four independent experiments (* correspond to significant ($p < 0.05$) difference in mode count associated with cells incubated with serotonin and serotonin in the presence of genistein in cholesterol-replenished condition, relative to control cells). Representative confocal microscopic images of cholesterol-replenished (subsequent to statin treatment) HEK-5-HT$_{1A}$R cells labeled with anti-myc antibody Alexa Fluor 488 conjugate (green) and (c) transferrin Alexa Fluor 568 conjugate or (d) cholera toxin B Alexa Fluor 594 conjugate (red). The scale bar represents 10 μm. (e) The extent of colocalization between serotonin$_{1A}$ receptor and transferrin (or cholera toxin) estimated using Manders' colocalization coefficient (M) under cholesterol-replenished condition. Data represent means ± SE of at least 9 independent measurements (** corresponds to significant ($p < 0.01$) difference in Manders' colocalization coefficient associated with colocalization of the serotonin$_{1A}$ receptor with cholera toxin B relative to transferrin under cholesterol-replenished conditions). See Methods for other details.

cells (not treated with lovastatin) and in cells treated with lovastatin alone (in absence of serotonin). On the other hand, upon treatment with serotonin, we observed internalization of the receptor to a similar extent under control as well as statin-treated conditions (Figure 1d,f). Taken together, these results suggest that chronic depletion of cholesterol utilizing lovastatin does not significantly alter the magnitude of agonist-induced internalization of the serotonin$_{1A}$ receptor.

Chronic Cholesterol Depletion Induces Switch in the Mechanism of Serotonin$_{1A}$ Receptor Endocytosis. Next, we explored the mechanism of endocytosis adopted by the receptor under statin-treated conditions. For this, we treated HEK-5-HT$_{1A}$R cells with inhibitors specific for clathrin- and caveolin-mediated endocytosis. We have previously demonstrated that the serotonin$_{1A}$ receptor predominantly internalizes via clathrin-mediated endocytosis upon treatment with serotonin.[45] Our previous results showed that pitstop 2 (inhibitor of clathrin-mediated endocytosis)[64] inhibited the endocytosis of the serotonin$_{1A}$ receptor, but genistein (inhibitor for caveolin-mediated endocytosis)[65] did not significantly inhibit the process.[45] Interestingly, as shown in Figure 2a, under conditions of statin-induced chronic cholesterol depletion, we did not observe a significant change in the internalization of the serotonin$_{1A}$ receptor upon treatment with pitstop 2. On the other hand, we observed that treatment with genistein resulted in appreciable inhibition

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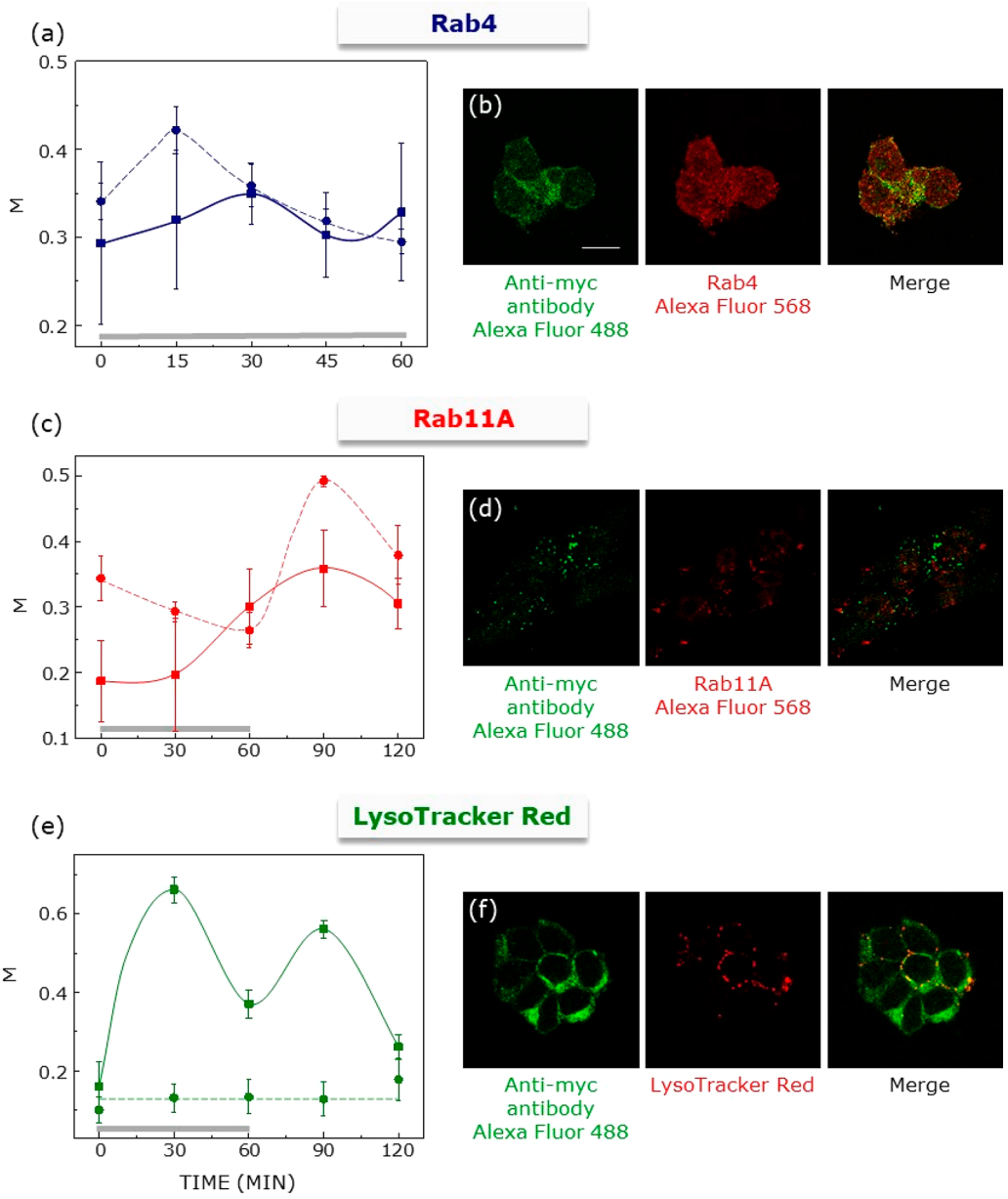

Figure 5. The serotonin$_{1A}$ receptor switches its intracellular trafficking pathway to lysosomal degradation under statin-treated conditions. Cells were incubated with 10 μM serotonin for different exposure times subsequent to statin treatment. The figure shows quantitative estimates of colocalization of the serotonin$_{1A}$ receptor with specific markers (a) Rab4, for early recycling endosomes, (c) Rab11A, for late recycling endosomes, and (e) LysoTracker, for lysosomes, analyzed using Manders' colocalization coefficient (M) under statin-treated conditions (■, solid lines). Lines connecting data points are provided merely as viewing guides. The horizontal gray bar indicates the duration of incubation with serotonin. The corresponding values of Manders' colocalization coefficient for untreated (control) cells are plotted for reference (●, dotted lines). Data represent means ± SE of at least 5 independent measurements. Representative confocal microscopic images showing colocalization between serotonin$_{1A}$ receptors (green) and (b) Rab4 after 30 min of incubation with serotonin, (d) Rab11A after 30 min of chase post serotonin incubation, and (f) LysoTracker, after 30 min of chase post serotonin incubation (red) are shown. The scale bar represents 10 μm. See Methods for other details.

in the internalization of the receptor upon chronic cholesterol depletion (see Figure 2a). Control experiments showed that the viability of HEK-5-HT$_{1A}$R cells was not compromised upon treatment with pitstop 2 and genistein subsequent to statin treatment (see Figure S1).

To further validate these observations, we monitored the colocalization of the serotonin$_{1A}$ receptor with known markers for clathrin- and caveolin-mediated endocytosis. Representative two channel confocal microscopic images of the serotonin$_{1A}$ receptor and transferrin (marker for clathrin-mediated endocytosis)[66] and cholera toxin B (marker for caveolin-mediated endocytosis)[67] are shown in Figure 2b,c. In our previous work, we demonstrated that upon agonist-induced internalization, the serotonin$_{1A}$ receptor exhibits higher colocalization with transferrin relative to cholera toxin B under normal conditions.[45] As shown in Figure 2d, under statin-treated conditions, we observed that the serotonin$_{1A}$ receptor exhibited higher colocalization (quantified using Manders' colocalization coefficient, M) with cholera toxin B relative to transferrin. Taken together, these results clearly demonstrate a switch in the mechanism of endocytosis of the

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serotonin$_{1A}$ receptor from clathrin- to caveolin-mediated endocytosis upon chronic cholesterol depletion.

Switch in the Mechanism of Internalization Due to Chronic Cholesterol Depletion Is Specific to the Serotonin$_{1A}$ Receptor. In order to test whether the observed switch in the mechanism of internalization upon treatment with statin is specific to the serotonin$_{1A}$ receptor, we explored the effect of chronic cholesterol depletion on the mechanism of internalization of transferrin and cholera toxin B (markers for clathrin- and caveolin-mediated endocytosis). As shown in Figure 3a,b, we observed a significant reduction in internalization of transferrin upon treatment with pitstop 2 (but not upon treatment with genistein) under both control and cholesterol-depleted conditions. Similarly, cholera toxin B internalization was inhibited by genistein (but not by pitstop 2) in control as well as statin-treated conditions (see Figure 3c,d). These results suggest that chronic cholesterol depletion does not alter the mechanism of internalization of known markers for clathrin- and caveolin-mediated endocytosis. In addition, these results, along with the results shown in Figure 2, suggest that the cholesterol-induced switch observed in the mechanism of internalization is a specific feature of endocytosis of the serotonin$_{1A}$ receptor.

Switch in the Mechanism of Internalization of the Serotonin$_{1A}$ Receptor Due to Chronic Cholesterol Depletion Is Reversible in Nature. We further explored the nature of this switch in the mechanism of internalization of the serotonin$_{1A}$ receptor from clathrin- to caveolin-mediated endocytosis upon chronic cholesterol depletion, by checking its reversibility. For this purpose, we metabolically replenished cholesterol in statin-treated HEK-5-HT$_{1A}$R cells by washing off the statin-containing medium and incubating the cells with medium containing 20% serum. Figure 4a shows that incubation of statin-treated cells in medium containing serum replenishes cholesterol to normal levels. We then monitored the endocytic machinery involved in the internalization of the serotonin$_{1A}$ receptor upon replenishment of cholesterol. As shown in Figure 4b, upon metabolic replenishment of cholesterol, pitstop 2 significantly inhibited the internalization of the serotonin$_{1A}$ receptor. However, treatment with genistein did not alter the extent of endocytosis of the receptor.

We further studied the colocalization of the serotonin$_{1A}$ receptor with transferrin and cholera toxin B upon cholesterol replenishment. Representative two-channel confocal microscopic images of the serotonin$_{1A}$ receptor and labeled transferrin or cholera toxin B are shown in Figure 4c,d. Quantitative analysis of colocalization showed that upon metabolic replenishment of cholesterol, the serotonin$_{1A}$ receptor exhibits significantly higher colocalization with transferrin relative to cholera toxin B (see Figure 4e). Taken together, these results exhibit a trend similar to our previous observations on the inhibition of serotonin$_{1A}$ receptor endocytosis by pitstop 2 (but not by genistein) and higher extent of colocalization with transferrin (relative to cholera toxin B) under normal conditions.[45] Our results suggest that the mechanism of serotonin$_{1A}$ receptor internalization is restored to clathrin-mediated endocytosis upon replenishment of cholesterol, thereby highlighting the reversible nature of the statin-induced switch from clathrin- to caveolin-mediated endocytosis.

Statin-Induced Chronic Cholesterol Depletion Alters the Intracellular Trafficking Pathway of the Serotonin$_{1A}$ Receptor. For exploring the intracellular fate of the serotonin$_{1A}$ receptor subsequent to its internalization via caveolin-mediated endocytosis under statin-treated conditions, we quantitatively monitored the colocalization of the receptor with specific endosomal markers at different time points of exposure to serotonin. We measured the colocalization of the receptor with markers for the early recycling endosome (Rab4),[68,69] late recycling endosome (Rab11A),[70,71] and lysosome (LysoTracker). As previously shown by us, the serotonin$_{1A}$ receptor exhibits colocalization with the early and late recycling endosomal markers upon internalization, but does not colocalize with the lysosomal compartment under normal conditions.[45] Figure 5a shows that upon statin treatment, the extent of colocalization of the serotonin$_{1A}$ receptor with Rab4 exhibits reduction. As shown in Figure 5c, we observed a similar reduction in the extent of colocalization of the serotonin$_{1A}$ receptor with Rab11A upon treatment with statin, as compared to untreated cells. Interestingly, in contrast to our observation on the lack of colocalization of the serotonin$_{1A}$ receptor with LysoTracker under normal conditions, we observed a significant colocalization of the receptor with the lysosomal compartment after 30 min of incubation with serotonin and after 30 min of chase (in serum-free medium) following 60 min of incubation with serotonin (see Figure 5e). Importantly, the time points of enhanced colocalization with lysosomes observed upon statin treatment correspond to the peaks observed in colocalization of the serotonin$_{1A}$ receptor with early and late recycling endosomes, respectively, in untreated conditions. Representative confocal microscopic images showing colocalization with Rab4 (at 30 min), Rab11A (at 90 min), and LysoTracker (at 90 min) are shown in Figure 5b,d,f, respectively.

We further validated these observations by measuring the plasma membrane receptor population after 60 min of chase (in serum-free medium) following 60 min of incubation with serotonin, under statin-treated conditions utilizing our flow cytometric assay. As shown in Figure 6, we observed a

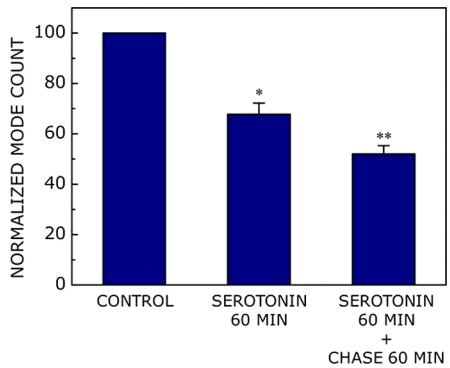

Figure 6. Plasma membrane population of the serotonin$_{1A}$ receptor exhibits reduction due to lysosomal degradation. HEK-5-HT$_{1A}$R cells treated with 5 μM statin for 24 h were incubated with 10 μM serotonin for 60 min and chased for 60 min in serum-free medium following serotonin incubation. Cells were subsequently fixed, and the plasma membrane receptors were labeled with anti-myc antibody Alexa Fluor 488 conjugate. Values are normalized to mode count associated with control cells. Data represent means ± SE of at least three independent experiments (** and * correspond to significant ($p < 0.01$ and $p < 0.05$) difference in mode count associated with cells incubated with serotonin upon statin treatment relative to control cells). See Methods for other details.

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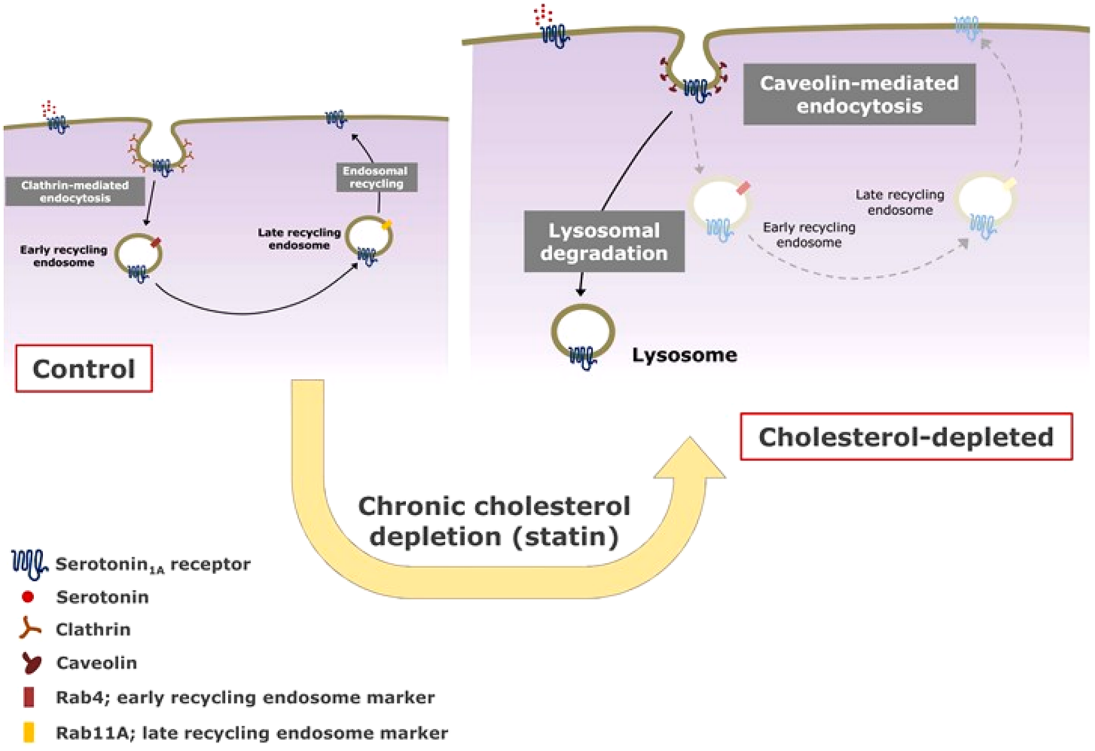

Figure 7. Cholesterol-induced switch in the endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor. A schematic representation of agonist-induced endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor under control and chronic cholesterol-depleted conditions. Upon statin treatment, the serotonin$_{1A}$ receptor undergoes a switch in the mechanism of internalization from clathrin- to caveolin-mediated endocytosis. Subsequent to internalization under statin-treated conditions, a significant proportion of the endocytosed receptors are routed toward lysosomal degradation, with a considerable reduction in the component undergoing membrane recycling. See text for more details.

reduction (relative to control) in the plasma membrane receptor population at this time point, indicating lysosomal degradation of the internalized receptor pool. Taken together, these results allow that statin-induced chronic cholesterol depletion results in a switch in the intracellular trafficking itinerary of the serotonin$_{1A}$ receptor from recycling to lysosomal degradation.

DISCUSSION

Cholesterol is one of the most extensively studied membrane lipids in terms of its effects on the function of GPCRs.[27−34] However, the role of cholesterol in regulating the endocytosis and intracellular trafficking of GPCRs remains largely unexplored. Extensive work from our laboratory has established the role of membrane cholesterol in the organization, dynamics, function, and oligomerization of the serotonin$_{1A}$ receptor, a representative neurotransmitter GPCR implicated in neuronal development and neuropsychiatric disorders.[46−55] We have recently demonstrated that the serotonin$_{1A}$ receptor exhibits agonist (serotonin)-induced internalization by clathrin-mediated endocytosis and subsequently recycles to the plasma membrane via endosomal recycling.[45] In the present work, we have explored the role of membrane cholesterol in the endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor by chronic depletion of cholesterol using lovastatin. Our results suggest that chronic cholesterol depletion by statin treatment induces a switch in the mechanism of internalization of the serotonin$_{1A}$ receptor from clathrin- to caveolin-mediated endocytosis, subsequently rerouting the internalized receptors predominantly toward lysosomal degradation instead of recycling (see Figure 7).

Acute depletion of cholesterol using MβCD has previously been employed as a common strategy to modulate membrane cholesterol.[60] Treatment with MβCD was reported to significantly reduce the rate of internalization of the transferrin receptor along with accumulation of flat clathrin-coated membranes and reduction in deep clathrin-coated pits.[72] In addition, MβCD has been shown to inhibit the endocytosis of transferrin and epidermal growth factor, which could be reversed upon replenishment of cholesterol.[73] On the contrary, acute cholesterol depletion by MβCD was shown to enhance the kinetics of internalization of the nicotinic acetylcholine receptor.[74,75] In case of GPCRs, acute cholesterol depletion has been reported to affect the endocytosis of δ-opioid receptor 1,[76] LPA$_1$ lysophosphatidic acid receptor,[77] and melanocortin-4 receptor.[78] Depletion of membrane cholesterol by MβCD was shown to inhibit the association of LPA$_1$ receptor with β-arrestin and subsequently clathrin-mediated endocytosis of the receptor. However, such cholesterol dependence of β-arrestin interaction with the receptor was not observed in case of M$_1$ muscarinic acetylcholine receptor and β_2-adrenergic receptor.[77]

Although treatment with MβCD offers a convenient approach to deplete membrane cholesterol, it is associated with several limitations owing to the acute nature and short duration of treatment.[60,61,79] On the other hand, chronic depletion of cholesterol utilizing statin offers a slow, physiologically relevant and metabolic approach to deplete cholesterol.[56,62,63] We therefore explored the role of cholesterol in the endocytosis of the serotonin$_{1A}$ receptor upon statin-mediated chronic cholesterol depletion. Our results show that although chronic cholesterol depletion does not

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significantly alter the extent of internalization of the serotonin$_{1A}$ receptor, statin treatment induces a switch in the pathway of internalization of the receptor from clathrin- to caveolin-mediated endocytosis. Importantly, metabolic replenishment of cholesterol could restore the mechanism of internalization to clathrin-mediated endocytosis (as observed under normal conditions). It is noteworthy that a switch in the mechanism of internalization from caveolin- to clathrin-mediated endocytosis was reported in case of internalization of the endothelin receptor type A upon oxidation of membrane cholesterol using cholesterol oxidase.[80]

We further explored the effect of statin treatment on the intracellular trafficking pathway adopted by the serotonin$_{1A}$ receptor. Quantitative colocalization with known markers for recycling and lysosomal degradation showed that upon statin treatment, a significant fraction of the endocytosed receptors were routed toward lysosomal degradation. This is in contrast to recycling of internalized receptors observed in untreated (without statin) conditions as previously demonstrated by us.[45]

The sensitivity of GPCRs to membrane cholesterol could be attributed to either the specific interaction of cholesterol with the receptor or modulation of general physical properties of the membrane, or a combination of both.[28,34,81] In this backdrop, the role of membrane cholesterol in modulating GPCR endocytosis assumes significance. Interestingly, unique structural features of cholesterol such as the presence of a 3β-hydroxyl group and its ability to promote the formation of ordered domains have been suggested to support endocytosis[82] and the internalization of *Yersinia pseudotuberculosis* into host cells.[83] Moreover, cholesterol is known to induce membrane curvature,[84] an essential structural feature associated with the process of endocytosis.

Our present results on the role of membrane cholesterol in the endocytosis of the serotonin$_{1A}$ receptor are significant in the backdrop of previous reports on the context-dependence of serotonin$_{1A}$ receptor endocytosis in terms of cellular phenotype. It has been shown that the serotonin$_{1A}$ receptor exhibits agonist-induced internalization in neurons derived from the dorsal raphe nucleus (autoreceptors), but not in hippocampal neurons (heteroreceptors).[85,86] This is important since neuronal membranes are characterized by substantial lipid diversity, often associated with the inherent complexity involved in their functioning.[87,88] The large diversity in membrane lipid composition and the relative abundance of lipids in various cell (or tissue) types could influence the mechanism of receptor endocytosis and intracellular trafficking. The differences in cholesterol content across tissue and cell types are especially relevant in this context. For example, the central nervous system accounts for 2% of body mass, yet contains ~25% cholesterol in humans.[89,90] In addition, cellular cholesterol content has been suggested to exhibit age dependence[91] and is developmentally regulated.[92] Our results could therefore provide a possible mechanistic basis underlying the role of varying lipid composition across cell and tissue types on endocytosis of GPCRs.

Cholesterol is a crucial lipid in the nervous system and is implicated in several neurological disorders, some of which are due to defective metabolism of brain cholesterol.[90,93,94] From a clinical standpoint, defective cholesterol biosynthesis has been implicated in developmental disorders such as Smith−Lemli−Opitz syndrome (SLOS) and desmosterolosis.[93,95] Interestingly, we have previously demonstrated that the serotonin$_{1A}$ receptor exhibits impaired signaling in a cellular system that mimics SLOS.[96] We plan to explore the endocytosis and intracellular trafficking of the serotonin$_{1A}$ receptor under such clinically relevant conditions of defective cholesterol biosynthesis in our future work.

Endocytosis as a mechanism of desensitization of the serotonin$_{1A}$ receptor has been implicated in the therapeutic action of popular antidepressants (SSRIs) such as fluoxetine.[43,44] Interestingly, recent cohort studies suggest that the combination of SSRIs and statins could have superior antidepressant effects relative to SSRI treatment alone.[97,98] In another study, the simultaneous administration of fluoxetine and statin has been suggested to enhance the levels of serotonin in rat brain.[99] In this backdrop, our results on a cholesterol-induced switch in the endocytic and intracellular trafficking pathway of the serotonin$_{1A}$ receptor assume significance. We envision that our results could provide useful insight in developing novel therapeutic interventions that could tap into the modulatory role of membrane cholesterol in GPCR endocytosis.

METHODS

Materials. $MgCl_2$, $CaCl_2$, genistein, serotonin, and doxycycline were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium:nutrient mixture F-12 (Ham) (1:1) (DMEM/F-12), hygromycin, and fetal calf serum were obtained from Invitrogen/Life Technologies (Grand Island, NY). Bicinchoninic acid (BCA) assay reagent for protein estimation was obtained from Pierce (Rockford, IL). Lovastatin was obtained from Calbiochem (San Diego, CA). Anti-myc antibody Alexa Fluor 488 conjugate was purchased from Millipore (Bedford, MA). Pitstop 2, anti-Rab4 [EPR3042], and anti-Rab11A [EPR7587(B)] rabbit monoclonal antibodies and goat anti-rabbit IgG Fc Alexa Fluor 568 conjugated were obtained from Abcam (Cambridge, MA). $DiIC_{16}$, transferrin Alexa Fluor 568 conjugated, cholera toxin B Alexa Fluor 594 conjugated, LysoTracker Red, and Amplex Red cholesterol assay kit were purchased from Molecular Probes/Invitrogen (Eugene, OR). All other chemicals used were of the highest available purity. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Cell Culture. HEK-5-HT$_{1A}$R cells were grown as described earlier.[45] Briefly, cells were grown in DMEM/F-12 medium supplemented with 10% fetal calf serum and 250 μg/mL of hygromycin B at 37 °C in a humidified atmosphere with 5% CO_2. Receptor expression was induced by incubating cells in medium containing 1 μg/mL of doxycycline for 24 h prior to experiments.

Chronic Cholesterol Depletion Using Lovastatin and Metabolic Replenishment of Cholesterol. Chronic depletion of cholesterol from HEK-5-HT$_{1A}$R cells was achieved using lovastatin. Stock solution of lovastatin was prepared as described previously.[100] Cells grown for 24 h were treated with 5 μM lovastatin for 24 h in DMEM/F-12 medium with 10% serum at 37 °C. Subsequent to the treatment, medium containing lovastatin was removed, and excess statin was washed off with PBS. Metabolic replenishment of cholesterol was performed by incubating statin-treated cells with medium containing 20% serum for 24 h at 37 °C.

Estimation of Cholesterol Content. Free cholesterol content in lysates from HEK-5-HT$_{1A}$R cells was estimated using the Amplex Red cholesterol assay kit[101] and normalized to total cellular protein estimated using the BCA assay,[102] as described previously.[100]

Inhibition of Clathrin- And Caveolin-Mediated Endocytosis. Clathrin-mediated endocytosis was inhibited using 20 μM pitstop 2, and caveolin-mediated endocytosis was inhibited using 200 μM genistein, as described previously.[45]

Monitoring Receptor Endocytosis Utilizing Flow Cytometry. Endocytosis of the serotonin$_{1A}$ receptor was monitored utilizing a quantitative flow cytometric assay as described previously.[45] In brief,

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subsequent to treatment, HEK-5-HT$_{1A}$R cells were collected in PBS on ice, fixed, stained with anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution), and resuspended in PBS containing 2% serum. Data were acquired from 10,000 cells on a MoFlo XDP flow cytometer (Brea, CA) by exciting Alexa Fluor 488 at 488 nm and collecting emission using a 529/28 nm bandpass filter. Data analysis was performed on Summit analysis software version 5.4.0 to obtain mode count values corresponding to plasma membrane receptor population.

Confocal Microscopic Imaging To Monitor Receptor Endocytosis. HEK-5-HT$_{1A}$R cells plated on poly-L-lysine-coated glass coverslips were processed for confocal microscopic analysis of receptor endocytosis as described previously.[45] Anti-myc antibody Alexa Fluor 488 conjugate was used to label serotonin$_{1A}$ receptors, and the plasma membrane was labeled with DiIC$_{16}$.[45,103] In brief, cells were incubated with 8 μM DiIC$_{16}$ for 30 min on ice, fixed with 4% (w/v) formaldehyde for 10 min, and permeabilized with 0.5% (v/v) Triton X-100 for 5 min. Subsequently, cells were stained with anti-myc antibody Alexa Fluor 488 conjugate (1:100 dilution) for 60 min, washed, and mounted. Images were acquired on a Leica SP8 confocal microscope (Wetzlar, Germany). Alexa Fluor 488 was excited at 488 nm, and emission was collected between 500 and 560 nm. DiIC$_{16}$ was excited at 561 nm, and emission was collected between 570 and 640 nm. A 63×/1.4 NA oil immersion objective was used to acquire images under 1 airy condition.

Confocal Imaging of Serotonin$_{1A}$ Receptor Colocalization with Transferrin and Cholera Toxin B. Cells plated on poly-L-lysine-coated glass coverslips were processed for two-channel confocal microscopic imaging for quantifying colocalization between serotonin$_{1A}$ receptor (labeled with anti-myc antibody Alexa Fluor 488 conjugate) and transferrin (labeled with Alexa Fluor 568 conjugated) or cholera toxin B (labeled with Alexa Fluor 594 conjugated) as described previously.[45] In brief, cells were first labeled with anti-myc antibody Alexa Fluor 488 on ice and subsequently incubated with cholera toxin B Alexa Fluor 594 conjugate and 10 μM serotonin at 37 °C for 60 min. Cells were then incubated with 150 mM NaCl, 50 mM acetic acid solution for 15 min to remove cell surface-bound antibodies, fixed in 4% (v/v) formaldehyde, and mounted. Images of z-sections were acquired on a Leica SP8 confocal microscope using a 63×/1.4 NA oil immersion objective under 1 airy condition and a fixed step size of 0.5 μm. Anti-myc antibody Alexa Fluor 488 conjugate was excited at 488 nm, and emission was collected between 500 and 560 nm. Transferrin Alexa Fluor 568 conjugated and cholera toxin B Alexa Fluor 594 conjugated were excited at 561 and 594 nm, and emission was collected between 570 and 640 and 600−640 nm, respectively.

Quantitation of Transferrin and Cholera Toxin B Internalization. HEK-5-HT$_{1A}$R cells were incubated with 10 μg/mL transferrin Alexa Fluor 568 conjugated (or cholera toxin B Alexa Fluor 594 conjugated) in serum-free medium at 37 °C for 30 min. Cells were transferred to ice and washed twice with cold PBS to remove excess unbound transferrin (or cholera toxin B). Transferrin (or cholera toxin B) bound to plasma membranes was removed by incubating cells with 50 mM acetic acid, 150 mM NaCl solution for 15 min. Cells were then fixed with 4% (w/v) formaldehyde, washed with PBS, and mounted. Microscopy was performed on a Leica SP8 confocal microscope. z-Section images of transferrin Alexa Fluor 568 conjugate and cholera toxin B Alexa Fluor 594 conjugate from the entire cellular volume were acquired with a fixed step size of 0.5 μm using a 63×/1.4 NA oil immersion objective under 1 airy condition as described above. Internalized transferrin Alexa Fluor 568 conjugate (or cholera toxin B Alexa Fluor 594 conjugate) was quantified by counting their respective puncta using the Analyze Particles function of ImageJ (National Institutes of Health, Bethesda) within the diameter range of 0.2−1 μm. Number of puncta was normalized to area enclosed by the maximum intensity projection generated by merging all z-sections.

Monitoring Intracellular Trafficking of the Serotonin$_{1A}$ Receptor by Confocal Microscopy. Cells plated on poly-L-lysine-coated glass coverslips were processed for two-channel confocal microscopic imaging for colocalization between serotonin$_{1A}$ receptor (labeled with with anti-myc antibody Alexa Fluor 488 conjugate) and markers for early recycling endosomes (Rab4) (or late recycling endosomes (Rab11A) or lysosomes (LysoTracker)) as described previously.[45] Images of z-sections were acquired on a Leica SP8 confocal microscope using a 63×/1.4 NA oil immersion objective under 1 airy condition and a fixed step size of 0.5 μm. Anti-myc antibody Alexa Fluor 488 conjugate was excited at 488 nm, and emission was collected between 500 and 560 nm. Alexa Fluor 568 (conjugated to secondary antibody against Rab4 and Rab11A primary antibodies) and LysoTracker Red were excited at 561 nm, and emission was collected between 575 and 630 nm.

Colocalization Analysis. Colocalization between fluorescence signals from two channel confocal microscopic images was analyzed using Manders' colocalization coefficient (M),[104] as described previously.[45] Costes' method for automated thresholding was employed to determine threshold intensities of the two channels.[105] The JACoP plug-in[106] for ImageJ (National Institutes of Health, Bethesda) was used to analyze colocalization on a single section from a z-stack.

Statistical Analysis. Significance levels were analyzed using Student's two-tailed unpaired t-test using GraphPad Prism software, version 4.0 (San Diego, CA). Plots were generated using OriginPro software, version 8.0 (OriginLab, Northampton, MA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.9b00659.

Figure S1: Viability of HEK-5-HT$_{1A}$R cells under various treatment conditions (PDF)

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Author Contributions

G.A.K. performed experiments and analyzed data. G.A.K. and A.C. designed experiments. G.A.K. and A.C. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BCA, bicinchoninic acid; $DiIC_{16}$, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; GPCR, G protein-coupled receptor; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; MβCD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SSRI, selective serotonin reuptake inhibitor

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