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Docosahexaenoic Acid (DHA) Induced Morphological Differentiation of Astrocytes Is Associated with Transcriptional Upregulation and Endocytosis of β_2-AR

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Abstract

Docosahexaenoic acid (DHA), an important ω-3 fatty acid, is abundantly present in the central nervous system and is important in every step of brain development. Much of this knowledge has been based on studies of the role of DHA in the function of the neurons, and reports on its effect on the glial cells are few and far between. We have previously reported that DHA facilitates astrocyte differentiation in primary culture. We have further explored the signaling mechanism associated with this event. It was observed that a sustained activation of the extracellular signal-regulated kinase (ERK) appeared to be critical for DHA-induced differentiation of the cultured astrocytes. Prior exposure to different endocytic inhibitors blocked both ERK activation and differentiation of the astrocytes during DHA treatment suggesting that the observed induction of ERK-2 was purely endosomal. Unlike the β_1-adrenergic receptor (β_1-AR) antagonist, atenolol, pre-treatment of the cells with the β_2-adrenergic receptor (β_2-AR) antagonist, ICI-118,551 inhibited the DHA-induced differentiation process, indicating a downstream involvement of β_2-AR in the differentiation process. qRT-PCR and western blot analysis demonstrated a significant induction in the mRNA and protein expression of β_2-AR at 18–24 h of DHA treatment, suggesting that the induction of β_2-AR may be due to transcriptional upregulation. Moreover, DHA caused activation of PKA at 6 h, followed by activation of downstream cAMP response element-binding protein, a known transcription factor for β_2-AR. Altogether, the observations suggest that DHA upregulates β_2-AR in astrocytes, which undergo endocytosis and signals for sustained endosomal ERK activation to drive the differentiation process.

Keywords Astrocytes · β_2-Adrenergic receptor · Docosahexaenoic acid · Endocytosis · PKA · GPR120

Introduction

The long-chain fatty acid, docosahexaenoic acid (DHA) (22:6n-3), is much known for maintaining membrane fluidity and regulating membrane protein functions, which in turn supports signal transduction and neurotransmission of the brain cells [1–5]. DHA primarily acts as an endogenous ligand for several G-protein-coupled receptors (GPCR) like GPR 40, GPR 120, and retinoid X receptor (RXR) [6–8] and modulates cellular signaling via the activation of these GPCRs. Regulation of signal selectivity and specificity is highly complex leading to the activation of selective pathways that eventually elicit biological responses. DHA/GPR40 involves the PLC/IP3 signaling pathway and modulates intracellular Ca^{2+} mobilization during neuronal differentiation in rat [9], inducing downstream phosphorylation of CREB protein during adult neurogenesis in primates [10]. GPR120 mostly mediates the anti-inflammatory action of DHA and involves transforming growth factor-β-activated kinase 1 binding protein (TAB1) downstream of the signaling cascade [11]. The sensitivity of early developmental period toward DHA indicates its importance for proper brain development, especially for the cerebral cortex and retina [12]. Among the various brain cells, astrocytes are the only cells capable of synthesizing DHA in the brain [13–15], and cerebromicrovascular endothelial cells in the brain tend to cooperate with the astrocytes in synthesizing DHA [16, 17]. Nevertheless, neurons appear to be the main site for DHA accumulation [18–20], and a number of studies suggest that DHA has immense effect on neuronal development, promoting neurogenesis, neuritogenesis, neurite growth (both length and branching), and synaptogenesis in the newborn [21–23].

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On the other hand, information on the underlying role of DHA in astrocyte is scarce. Reports suggest that DHA favor the coupling of gap junction in astrocytes, by redistributing functional connexin-43 [24], regulate glutamate uptake [25], and promote glucose uptake and metabolism [26, 27]. DHA-enriched astrocytes are capable of alleviating stress by restoring the normal glutamate recycling in the corticosterone-induced stressed rodent brain and also resist stress-induced morphological changes in astrocytes by restoring the GFAP cytoskeleton [28]. Studies from this laboratory suggested that DHA facilitates astrocyte differentiation in vitro, characterized by changes in cell shape [29].

During morphogenesis of the brain, astrocytes have been found to undergo dynamic changes in morphology leading to matured astrocytes in the adult brain [30]. Such changes in morphology of astrocytes have been reported in vitro by this laboratory as well as by others in primary astrocyte cultures [31–34]. Such studies demonstrate two distinct morphological transformations of the cells; initially, the immature star-like radial glia differentiates into flat epithelioid polygonal cells which finally transform into a mature process-bearing cells. Each of these morphological forms appears to be critical for the progression of brain development [35–37]. While during early development, the immature radial glia helps in guiding neuronal migration, laminar pattern formation, and axonal guidance; mature astrocytes promote neurite extension and support various other supporting functions during subsequent developmental processes. Various second messengers including cAMP as well as their modulators such as growth factors are known to regulate astrocyte differentiation [38, 39]. In addition to DHA, thyroid hormones (TH) are also known to trigger such morphological differentiation in astrocytes [34, 40], and further studies suggest increased β-adrenergic transmission during both TH-induced and DHA-induced differentiation of astrocytes [29, 41].

Much of our knowledge on the interactions of DHA and adrenergic receptors comes from isolated studies in the heart tissue. DHA treated cardiomyocytes not only increased DHA content in membrane phospholipids but also exerted a positive influence on the β-AR transduction [42]. It has been observed from reperfusion studies on hypoxic heart that incorporation of DHA in membrane phospholipid is associated with decreased functional responses of α-AR and β-AR [43, 44], which may account for the cardiac protective effect of ω-3 PUFA. Both hydrocortisone and epinephrine caused an increase in DHA level in the heart sarcolemma accompanied by a decrease in the number of β-AR binding sites [45]. Decreased DHA levels in the brain, due to dietary deficiency of ω-3 fatty acid, causes hypertension in animals due to reduced hypothalamic β_1-AR levels [46]. Previous studies from this laboratory suggest that increased ^{125}I-PIN binding to β-AR during DHA supplementation in primary culture of astrocytes could be attributable to the increase in mRNA level of β-AR and the increased number of binding sites [29].

It is evident from the literature that protein kinases also have some regulatory role in the cell differentiation process. Previous studies from our laboratory have suggested that PKA-mediated signaling is essential in TH as well as isoproterenol-mediated astrocyte differentiation [41, 47]. DHA is also known to activate kinases to exert its neuroprotective as well as differentiative effect on cells [48, 49]. *N*-docosahexenoyethanolamine (synaptamide), a metabolite of DHA, functions as neurogenic factor for neural stem cell (NSC) differentiation, through a PKA-dependent pathway [48] as well as through PLC/IP3 pathway [9]. DHA mediate cAMP-dependent activation of PKA leading to activation of CREB via ERK, causing transcription of GFAP, S100β, the Na^+-dependent glutamate/aspartate transporter (GLAST), etc. that participate in astrocyte differentiation [50].

Two small G-proteins, Ras and Rap-1, along with some other scaffold proteins have been identified to mediate PKA-dependent ERK activation. PKA phosphorylation of Rap-1 is essential for its binding to adaptor protein which in turn links Rap-1 to scaffold protein Raf and initiates ERK signaling [51]. In neuron, ERK activation through PKA-induced Rap1/B-raf-dependent manner, that rescued cells from death and caused proliferation of cells but in astrocytes, as B-raf is absent [52]; Ras-Raf-1 pathway might be activated causing cessation of proliferation of cells [47]. PKA-dependent phosphorylation of Raf-1 has been shown to inhibit cellular proliferation in various cell types [53, 54].

With an aim to investigate how DHA regulates the responsiveness of β-AR to promote differentiation of the cells, the present study was conceived. Furthermore, it is well established that the duration of signals is critical for decisions concerning cell fate. A good example is ERK signaling which when activated transiently causes proliferation of cells but sustained activation of ERK leads to cell differentiation [47, 55–57]. Studies on neuron as well as astrocytes have confirmed that sustained ERK activation is necessary for the cells to undergo differentiation [41, 47, 58, 59]. Recent evidence from our laboratory suggests that such sustained induction in ERK signaling is endosomal in origin [60]. The ERK activity retained in the cytosol may phosphorylate specific cytoplasmic substrates helpful for cytoskeletal rearrangement [61] involved with morphogenesis and cell survival [62, 63]. We, therefore, undertook studies to determine whether the differentiation of astrocytes induced by DHA occurred due to a delayed but sustained activation of ERK involving endosomal signaling. We report here that DHA induces β_2-AR synthesis in the astrocytes which subsequently internalizes and triggers endosomal signaling, resulting in sustained pERK activation to drive the process of differentiation of the cells.

Materials and Methods

Preparation of Primary Cultures of Astrocytes from Rat Brain

Animals were handled following the guidelines of the Institutional Animal Care and Use Committee prepared according to that of the Indian National Science Academy. Animal experimentation was approved by the institutional animal ethics committee appointed by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of the animal welfare division under the Ministry of Environment and Forest, Government of India (Registration no. 147/CPCSEA).

Newborn Sprague-Dawley rat pups (< 24 h old) of any sex were collected randomly and sacrificed for primary culture of astroglia as reported earlier [41]. Briefly, from the neocortex part of the brain, single-cell suspension was prepared in DMEM (Dulbecco's modified Eagle's medium) (Gibco BRL) supplemented with 50 μg/ml gentamycin, 50 μg/ml streptomycin, sodium bicarbonate, and 10% TH-depleted FBS pH 7.4. It was previously reported by us that normal fetal calf serum contains thyroid hormones that can induce transformation of astrocytic cells [34]. The serum used in the present study, therefore, made TH deficient to rule out any interaction of the hormones as carried out by us earlier [60, 64, 65]. Repeated adsorption of the TH with Dowex 1 × 8 (200–400) mesh, Cl^- form (Sigma–Aldrich), causes significant decline in the serum TH levels [66] and was used as TH-depleted serum. To yield highly purified (95%) preparation of astrocytes, cells were initially plated in poly-L-lysine (Sigma–Aldrich) coated plates and kept for 5 min for preferential attachment of neurons. After two such attachments, astrocytes were finally seeded into fresh plates at 6×10^6 cells/90-mm plate and maintained in a Forma CO_2 incubator (5% CO_2/95% air) at 37 °C. Astrocytes grown on glass coverslips were used for immunocytochemical studies. Ten-day-old cultures, grown in 10% TH-deficient serum, were used for the study since the astrocytes at this stage remained in polygonal form and could not further differentiate into the mature process-bearing cells [34].

Drug Treatment

Media containing 1% TH-deficient serum with or without 100-μM DHA (Sigma–Aldrich), solubilized in 0.1% ethanol (Merck), were added to 10-day-old astrocytes culture and maintained for a further period of 48 h (time taken for completion of differentiation process). Such treatment conditions and the concentration of DHA have been previously shown to cause high incorporation of DHA in astrocyte producing maximum effect in respect to morphology change [29]. Further studies have shown that this concentration is safe for astrocyte and does not cause production of malondialdehyde (MDA), a harmful end product of lipid peroxidation [67]. Untreated controls received vehicle only. PD098059 and H-89 were used to inhibit ERK-2 and PKA activation respectively. AH 7614, DC 260126 (Tocris Bioscience), atenolol, and ICI-118,551(Sigma–Aldrich) were used for blocking GPR120, GPR40, β_1-AR, and β_2-AR respectively. Cycloheximide was used to inhibit the synthesis of new proteins in the cells. Dynasore (Enzo Life Science), bafilomycin A1(Alfa Aesar), and brefeldin A (Sigma–Aldrich) were dissolved in an appropriate solvent according to manufacturer's instruction and used for inhibiting dynamin, vacuolar-type H^+-ATPase (V-type), and nucleotide exchange factor for ADP-ribosylation factor (ARF) respectively.

Sample Preparation and Protein Extraction

Cells were rinsed with ice-cold 50-mM Tris-HCl buffer, pH 7.4 (TB) containing 0.9% NaCl, scraped into a small volume of TB, sonicated, and centrifuged at 40,000*g* for 15 min. The supernatant containing cytosolic proteins was collected and the pellet was re-suspended in fresh lysis buffer. For immunoprecipitation experiments, both pellets and supernatants (as cytosol) were used. Protein concentrations were determined [68].

Immunoprecipitation and Western Blot Analysis

Membrane pellets and supernatant obtained, as described earlier, were subjected to immunoprecipitation followed by immunoblotting against β_2-AR (Santa Cruz) as published earlier [60].

Western blot analysis was carried out in whole cell lysate dissolved in Laemmli buffer. The lysates were subjected to 10% SDS-PAGE followed by transfer to PVDF membranes (Millipore). Blocking of non-specific binding was carried out by treating the blots with 5% nonfat dry milk in TBS for 1 h at 37 °C. Expression of individual proteins was evaluated using antibodies β-ARK-1, β-arrestin-1, β-arrestin-2, phosphorylated β-arrestin-1(Santa Cruz), pERK-2, and pCREB (cell signaling) followed by incubation with an HRP-conjugated secondary antibody. Blots were revealed by a chemiluminescent reagent (GE Healthcare). For loading control, the same blots were immunostained with anti-ERK (ERK-2). All the bands were analyzed densitometrically using the ImageJ 1.29 software.

RNA Isolation and cDNA Preparation

Total RNA from cultured astroglial cells was isolated using Trizol™ (Invitrogen) following the manufacturer's instructions. Briefly, cells were taken in a tube containing 1-ml Trizol™ and sheared by pipetting. To this, 300-μl chloroform (Spectrochem) was added and the contents mixed by inverting

the tube several times. After standing for 5 min, the tube was centrifuged at 12,000 ×*g* for 15 min. The upper aqueous layer was collected very carefully, mixed with 500 μl of isopropanol (Merck) and kept for 15 min to precipitate RNA. After centrifuging at 16,000 ×*g* for 15 min, the resultant pellet was washed twice with 75% chilled ethanol (Merck), vacuum dried, dissolved in DEPC (Sigma–Aldrich, St. Louis, USA) treated water, and quantified spectrophotometrically at OD 260 nm.

RevertAid First Strand cDNA Synthesis Kit (Fermentas) was used to prepare cDNA from 1 μg of the total RNA. Briefly, the RNA sample was mixed with oligo(dT) and incubated for 5 min at 65 °C. Then mixture of dNTPs, reverse transcriptase (RT), and RT reaction buffer was added to the RNA to start the reaction. The reaction was stopped by heating at 70 °C for 5 min to inactivate RT enzyme and immediately chilled on ice.

Real-Time PCR

To quantitate the levels of mRNA, RT products were analyzed by real-time RT-PCR using similar cycling condition in Applied Biosystems StepOne ™ system. PCR was carried out in triplicate in the presence of Maxima SYBR Green qPCR master mix (2X) (Thermo Fisher) using 0.5 pmol of forward and reverse primer (Table 1) and 1-μl RT product in 20-μl final reaction mixture according to manufacturer's protocol for 35 cycles. $2^{-\Delta CT}$ method was followed to calculate the relative mRNA levels and multiplying them by 100,000 or 1000, whichever is applicable, to get a whole number. The mRNA level for each sample was normalized against GAPDH mRNA as a reference gene. Data are presented as the ratio of relative expression of each transcript compared to the expression of the housekeeping gene, GAPDH.

Assay of cAMP-Dependent Protein Kinase or Protein Kinase A (PKA)

Cells, after proper washing, were harvested in extraction buffer (20-mM Tris-HCl, pH 7.2, 1-mM EGTA, 2-mM EDTA, 50-mM βME, 0.5-M theophylline, 2-mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors for PKA assay as reported earlier) [47]. Reactions were started with the addition of 10 μg of protein to the reaction mixture containing 20-mM Tris-HCl, pH 7.2, 1-mM EGTA, 50-mM NaF, 20-mM PNPP, 10-mM Mg-acetate, 20-μM [γ-^{32}P]-ATP (Bhaba Atomic Research Centre, Mumbai, India), and 100-μM kemptide substrate. Reactions were stopped with phosphoric acid, spotted in the P81 paper (Whatman International Ltd.) and washed three or four times in 75-mm phosphoric acid. Radioactivities retained on dry papers were measured by liquid scintillation counter, and results were expressed as the ratio of the two PKA activities in the absence and presence of 10-μM cAMP.

Immunocytochemistry

Cells grown on coverslip were fixed in ice-cold 4% paraformaldehyde for 10 min, washed three times with phosphate-buffered saline (PBS), and incubated with GFAP (Santa Cruz) in 1:10 dilution, followed by FITC-conjugated goat anti-mouse IgG (1:50 dilution) (Sigma–Aldrich). All incubations were carried out for 30–45 min at 37 °C. The coverslips were washed with PBS, mounted onto glass slides in mounting medium, and examined under Leitz fluorescence microscope.

Morphometric Analysis

The morphometric analyses of astrocyte stellation were performed using Image J software. The changes in cross-section area (the basal area of a single cell) and perimeter (length of the outline of a single cell) of the cells were further correlated in terms of cell shape factor (CSF). Individual cells were identified based on their staining with default settings for brightness, contrast, etc. The parameter like area, perimeter, and integrated density was selected manually. A single cell was selected and its basal area was measured by using the software. CSF can be given by the equation,

$$\mathrm{CSF} = 4\pi A/P^2.$$

A, cross-section area; *P*, perimeter of the cell.

The GFAP expression (corrected total cell fluorescence—CTCF) was quantified by measuring the green fluorescence with Image J and CTCF were calculated by the formula,

$$\mathrm{CTCF} = \text{integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings}).$$

Statistical Analysis

The data obtained were statistically calculated using Student's *t* test and one-way analysis of variance (ANOVA), followed by Tukey's test.

Results

Effect of DHA on the Differentiation of Astrocytes and on ERK Signaling

Upon exposure to DHA up to 48 h, flat polygonal astrocytes differentiated into long process bearing matured cell (Fig. 1ai) as demonstrated by immunocytochemical analysis of cell morphology with GFAP. However, prior addition of PD098059 (50 μM), an upstream inhibitor of ERK prevented

Table 1 List of forward and reverse primers

Gene	Forward primer	Reverse primer	Product length	Annealing temp.
GPR40	5′-CCTTTGCACTA GGCTTTCCA-3′	5′-AGGTCAGAGC AGGCCAAAT-3′	115-bp	60 °C
GPR120	5′-TTCTCGGATG TCAAGGGCGAC-3′	5′-ATAACCAGGTCC TGCCGGAAGTT-3′	812-bp	60 °C
β_1-AR	5′-ATGTGTGACG GCCAGCATCG-3′	5′-AAGCGGCGCT CGCAGCTGTC-3′	374-bp	58.5 °C
β_2-AR	**5′-**GAGACCCTG TGCGTGATTGC-3′	**5′-**GAGGACCTT CGGAGTCCGTG-3′	407-bp	57.5 °C
GAPDH	5′-TGGGGTGATG CTGGTGCTGAG-3′	5′-GACATGCCGC CTGGAGAAACC-3′	497-bp	51.8 °C

the differentiation induced by DHA (Fig. 1bi, right panel) suggesting an important contribution of ERK in this process. At that concentration, PD098059, by itself, had no effect on the cell morphology (Fig. 1bi, left panel). The differentiation of astrocytes is characterized by a change in cell morphology including decrease in cross-section area and increase in perimeter as well as an increase in GFAP expression. The morphometric analysis of cells revealed the significant decrease in cross-section area and increase in perimeter of the cell (Fig. 1aiii) and also showed an increase in GFAP expression (Fig.

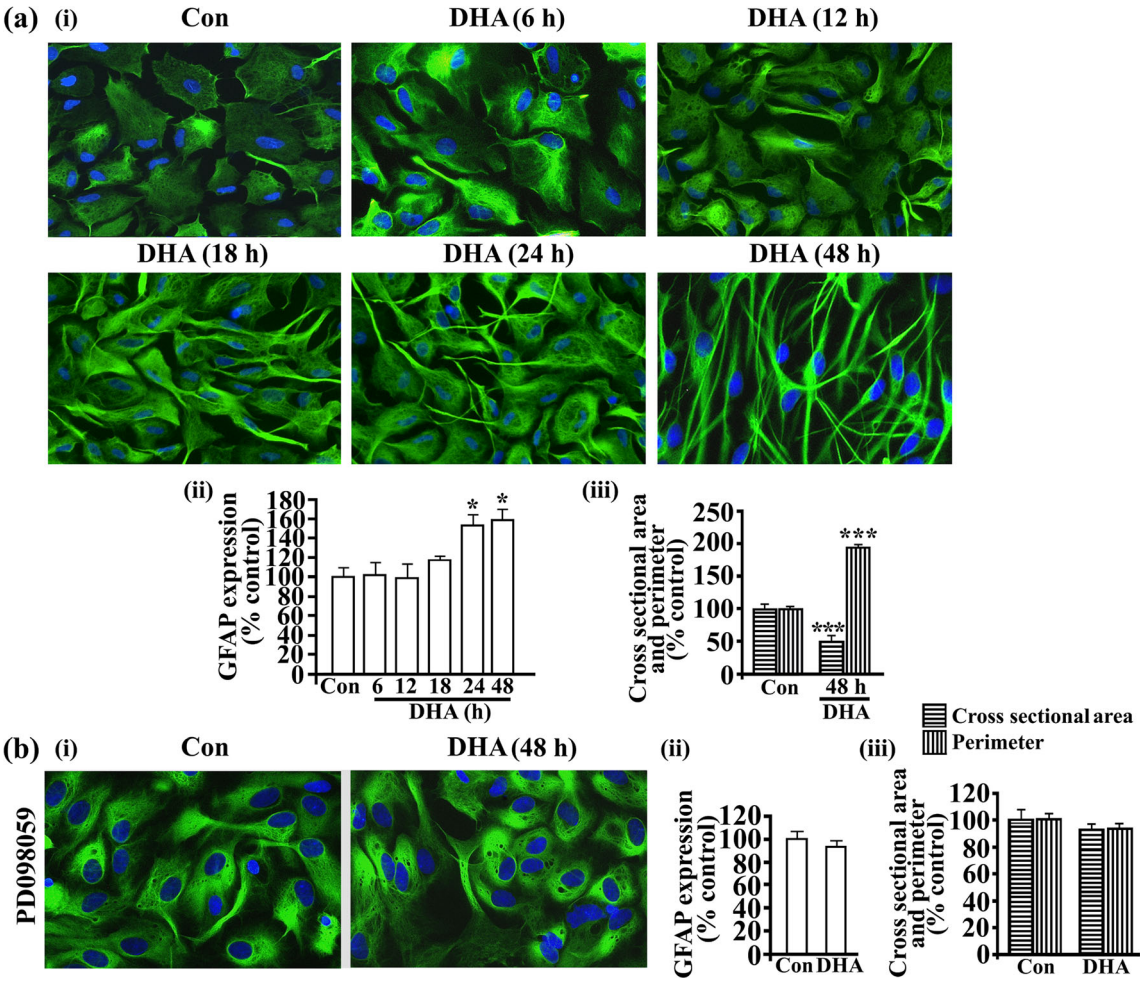

Fig. 1 Effect of DHA on morphological differentiation of astrocyte. The 10-day-old astrocytes were treated with or without DHA (100 μM) for different time up to 48 h. (ai) Morphology of cells was revealed by immunocytochemical staining using anti-GFAP antibody followed by FITC-conjugated IgG (green). Hoechst 33342 (blue) was applied to stain the nucleus. (aii) The expression of GFAP at different time periods was analyzed. (bi) In another set of experiment, the cultures treated with PD098059 (50 μM) prior to exposure to DHA were immunostained and (bii) GFAP expression was measured. Relative changes in cell cross-section area and perimeter were quantified for (aiii) DHA treated as well as (biii) PD098059 treated cell. *$p < 0.05$ and ***$p < 0.001$ indicate statistical differences from control

1aii), upon treatment with DHA, while PD098059 failed to induce any changes in cross-section area, perimeter (Fig. 1biii), and GFAP expression (Fig. 1bii) of astrocytes. The CSF also indicates the stellation of the astrocytes treated with DHA (Table 2). Western blot analysis of pERK-2 levels during treatment of the astrocytes with DHA, at different time points, indicated a significant and sustained induction in ERK-2 activity by DHA, from 6 to 48 h (Fig. 2a).

An important downstream event of the ERK pathway is the phosphorylation of the CREB, a transcription factor having an important role in regulating cell morphology as suggested by the previous report of others and from our laboratory [47, 69]. Immunoblots, using antibody specific to pCREB, showed a significant increase in pCREB levels with peak activity at 12 h (Fig. 2b). Results suggest that activation of ERK-2 and its downstream CREB signaling is critical for DHA-induced cell differentiation.

Table 2 Morphometric analysis of astrocyte shape at different condition

Serial no.	Treatment given	Condition	CSF	*n*
1		Control	0.67	12
		DHA treated	0.09	14
2	PD098059	Control	0.60	7
		DHA treated	0.66	12
3	Dynasore	Control	0.65	10
		DHA treated	0.62	10
4	Bafilomycin A1	Control	0.58	9
		DHA treated	0.63	12
5	Brefeldin A	Control	0.61	9
		DHA treated	0.60	11
6	Atenolol	Control	0.63	13
		DHA treated	0.09	12
7	ICI-118,551	Control	0.68	12
		DHA treated	0.69	14
8	Cycloheximide	Control	0.73	11
		DHA treated	0.72	11
9	H-89	Control	0.65	11
		DHA treated	0.67	11
10	AH 7614	Control	0.65	10
		DHA treated	0.68	13
11	DC 260126	Control	0.69	13
		DHA treated	0.09	12
12		Control	0.58	11
		DHA treated (for 6 h only)	0.1	11

CSF was calculated according to the equation: $4\pi A/P2$, where A is the cell cross-section area and P the cell perimeter. This measure ranges from 0 to 1 indicating a line and a perfect circle (one-way ANOVA compared with control, $^{***}p < 0.001$). "n" denotes the number of cells measured. The cells are taken from different field of at least three or four experiments or culture

Effect of DHA on Endosomal Signaling

A prolong or sustained activation of MAPK is attributed to endosomal signaling, as discussed earlier [70, 71]. Using pharmacological inhibitors of endocytosis like Dynasore, bafilomycin A1, brefeldin A, we determined whether the observed activation of ERK-2 during exposure of the astrocytes to DHA was a sequel of endosomal signaling. Western blot analysis revealed that all these endocytosis inhibitors abolish the ERK-2 activation following DHA treatment (Fig. 3ai, bi, ci), but these inhibitors itself had no effect on ERK-2 activation. Interestingly, DHA-induced differentiation of astrocytes is also blocked by these inhibitors (Fig. 3aii, bii, cii) with no significant changes in cell cross-section area, perimeters (Fig. 3aiii, biii, ciii), and GFAP expression (Fig. 3aiv, biv, civ). Taken together, our observations suggest that the sustained activation of ERK-2 during DHA-induced differentiation appears to involve endosomal signaling.

DHA-Induced Differentiation Involved β_2-AR System

It is a general consensus that the endocytic pathway tightly controls the activity of G-protein-coupled receptors (GPCRs) [72–74]. The functional role of the much known GPCR, the β-AR system in the morphological transformations of astrocytes, is well established [40, 41]. We observed that prior exposure of the 10-day-old astrocyte cultures to the β_1-AR antagonist, atenolol, (Fig. 4a) could not block the DHA-induced morphological changes of astrocytes (Fig. 4ai, right panel). Significant changes in cross-section area and perimeter of the cell (Fig. 4aii), as well as GFAP expression (Fig. 4aiii), could be observed after treatment with atenolol. On the contrary, exposure of β_2-AR antagonist, ICI-118,551 (Fig. 4b) completely blocked the changes in cross-section area and perimeter of the cell (Fig. 4bii) along with GFAP expression (Fig. 4biii), thereby, blocking the transformation of the flat polygonal cell to process bearing astrocytes (Fig. 4bi, right panel). These antagonists, used in the study, had no effect on the cell morphology when given alone (Fig. 4ai, 4bi, left panel). The results suggest the possibility of the involvement of β_2-AR in astrocyte differentiation.

Additionally, quantitative analysis of the mRNA and protein expression levels of both isoforms of β-AR during exposure of the cells to DHA was also carried out. Although β_1-AR mRNA levels showed a significant decrease during the entire period of DHA treatment, from 6 to 48 h (Fig. 4aiv), no significant alterations in the corresponding protein levels were observed (Fig. 4av). On the other hand, quantitation of β_2-AR mRNA by qRT-PCR analysis showed that there was a significant upregulation of β_2-AR at 12 and 18 h of DHA treatment which came down to basal level by 24 h (Fig. 4biv), and the same trend was also observed in the expression of the β_2-AR protein (Fig. 4bv) as revealed by western blot

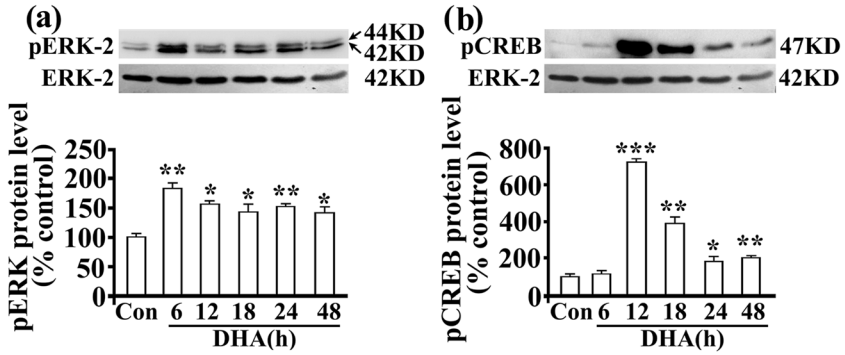

Fig. 2 Effect of DHA on pERK-2 and pCREB levels. The 10-day-old astrocytes were treated with DHA for different time (6–48 h), and total cell lysate was subjected to western blot analysis using (a) pERK-2 and (b) pCREB antibodies. Densitometric analysis of the blots is provided as histograms and expressed as mean ± SEM of independent treatments per group ($N = 3$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical differences from control. N denotes number of experiments performed

analysis. Overall, these observations indicate an involvement of β_2-AR in the DHA-induced morphological differentiation process.

Effect of DHA on β_2-AR Synthesis and Endocytosis

Like most GPCRs, β-ARs are subjected to endocytosis. To ascertain whether the β-ARs were undergoing endocytosis during DHA treatment, we determined the level of both isomeric forms of β-ARs, in the membrane as well as in the cytosolic fractions of the cells, in the absence and presence of DHA. Compared to untreated controls, immunoprecipitation followed by western blotting analysis revealed that there were no changes in the β_1-AR levels in the membrane (Fig. 5ai) as well as in cytosol (Fig. 5aii) when exposed to DHA. On the other hand, β_2-AR levels were significantly upregulated at 18 and 24 h in both the membrane (Fig. 5bi) and the soluble fractions (Fig. 5bii). Interestingly, in the presence of the endocytic inhibitor, Dynasore, the above increase in β_2-AR expression in the cytosol at 18 and 24 h by DHA (Fig. 5cii) was completely blocked, whereas that in the membrane continued to show increased β_2-AR on exposure to DHA (Fig. 5ci). The observations suggest that the DHA-induced increase in the β_2-AR receptor level in the cytosol may be attributed to endocytosis of these receptors.

It is apparent that DHA increases the synthesis of β_2-AR in the astrocytes, due to enhanced transcription, and is reflected from their enhanced levels both in the membrane and in the cytosol. This was further supported from the observation that prior treatment with cycloheximide (4 μg/μl) prevented the DHA-induced increase in β_2-AR levels, both in the membrane and in the cytosol (Fig. 5di–ii). In addition, cycloheximide inhibited the DHA-induced morphological differentiation of the astrocytes as revealed by immunocytochemical staining (Fig. 5ei) and also prevented any changes in the cell area, perimeter (Fig. 5eii), and GFAP expression (Fig. 5eiii). Taken together, our observations suggest that increased receptor synthesis by DHA is essential for endocytosis of more number of the receptor, which is essential for morphological differentiation of astrocytes.

Effect of DHA on Different Endocytosis Regulatory Proteins like β-ARK and β-Arrestin

Phosphorylation, desensitization, sequestration, resensitization, etc. are the key events during the process of GPCR endocytosis and some proteins like β-adrenergic receptor kinase (β-ARK), and arrestin families of protein largely regulate these events [75]. We undertook further studies to determine whether DHA might have some effect on these regulators resulting in the observed endocytosis of β_2-AR. Western blot analysis showed that there was no significant alteration in any of the arrestin isoforms namely, β-arrestin-1, β-arrestin-2, and β-ARK-1 levels up to 48 h of DHA treatment (Fig. 6a, b, d). To identify any changes in the active fraction of β-arrestin-1, we also measured the inactive form of β-arrestin-1 and p-β-arrestin-1, but observed no alteration after DHA treatment (Fig. 6c).

Role of PKA During DHA-Induced Differentiation of Astrocytes

Observations from the present study not only identified transcriptional activation of β_2-AR in astrocytes by DHA at 18–24 h, but also a peak activation of CREB at 12 h. The transcription of the β_2-AR gene is known to be facilitated by CREB, which binds to cAMP response element (CRE) in the promoter of the β_2-AR gene [76]. CREB activity is regulated upstream by PKA which in turn is activated by the stimulation of adenylyl cyclase and elevation of intracellular cAMP levels. We, therefore, analyzed the PKA activity in the cells from 2 to 48 h after addition of DHA, to ascertain whether activation of PKA by DHA could be related to the observed increase in CREB levels. Treatment of the astrocytes with DHA showed a significant increase in PKA activity

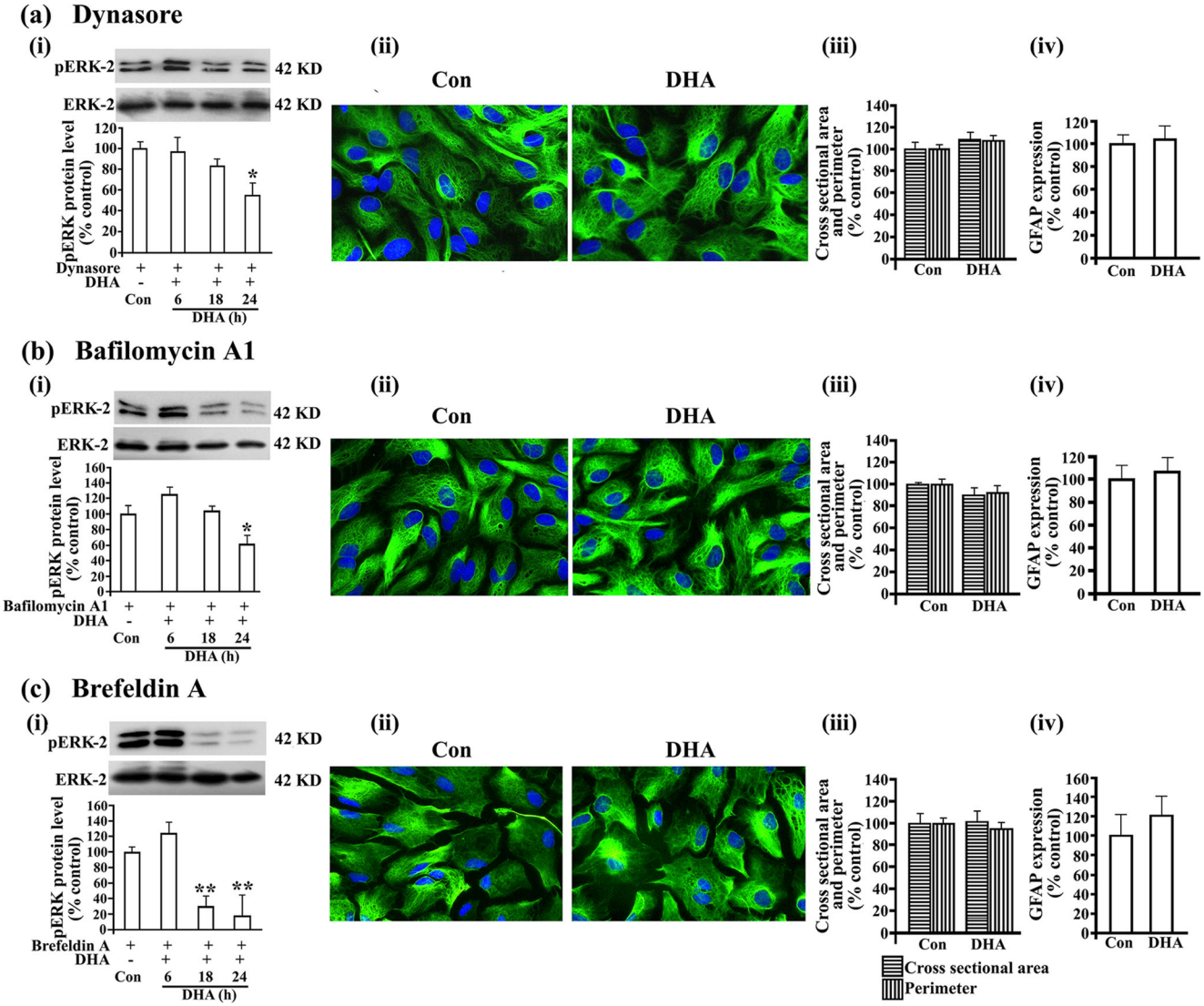

Fig. 3 Effect of endocytosis pathway inhibitors on ERK-2 activation as well as DHA-induced morphological differentiation of astrocytes. Ten-day-old astrocytes were treated with (a) Dynasore (80 μM), (b) bafilomycin A1 (20 nM), or (c) brefeldin A (10 μg/ml) for 30 min prior to addition of DHA for 48 h. Cells treated similarly but without DHA served as controls (con). (i) Total cell lysate was subjected to SDS-PAGE followed by western blotting against pERK-2 antibody and normalized against the internal loading control, ERK2. Densitometric histogram is expressed as mean ± SEM ($N = 3$). *$p < 0.05$ and **$p < 0.01$ indicate significantly different from vehicle-treated control. (ii) Morphology of cells analyzed by immunostaining with GFAP, followed by FITC-conjugated IgG (green) and Hoechst 33342 (blue) for nucleus. (iii) Changes in cross-sectional area, perimeter of cells, and (iv) GFAP expression were measured

(mean ratio of observed activity over total activity) at 6 h of treatment which returned to the basal level by 15 h (Fig. 7a).

To examine whether the increase in PKA activity following the addition of DHA could be causally linked to the maturation of the polygonal astrocytes, 10-day-old astrocyte cultures were treated with DHA initially for 6 h, and then, we maintained the cells in the absence of DHA up to 48 h. It was found that exposure of the astrocyte cells to DHA for the initial 6 h leading to activation of PKA was sufficient to induce transformation of the cells by 48 h (Fig. 7bi) characterized by increase in perimeter (Fig. 7biii) and GFAP expression (Fig. 7bii).

To examine, whether the increase in PKA activity had any effect on β_2-AR synthesis as well as astrocyte differentiation, we treated 10-day-old cells with PKA inhibitor, H-89 (100 nM), prior to DHA exposure. It was found that H-89 not only inhibited the DHA-induced synthesis of β_2-AR (Fig. 7d) but also blocked the differentiation of polygonal astrocytes to the mature cells (Fig. 7ci, right panel) with no alteration in GFAP expression (Fig. 7cii) and cell perimeter (Fig. 7ciii). H-89, by itself, had no effect on astrocytes morphology (Fig. 7ci, left panel). Application of H-89 could also block the sustained activation of ERK-2 (Fig. 7e) and activation of CREB (Fig. 7f). Taken together, these observations suggest that PKA may have an important contribution to the DHA-induced transformation of astrocytes, and CREB is involved as transcription factor in DHA-mediated transcription of β_2-AR.

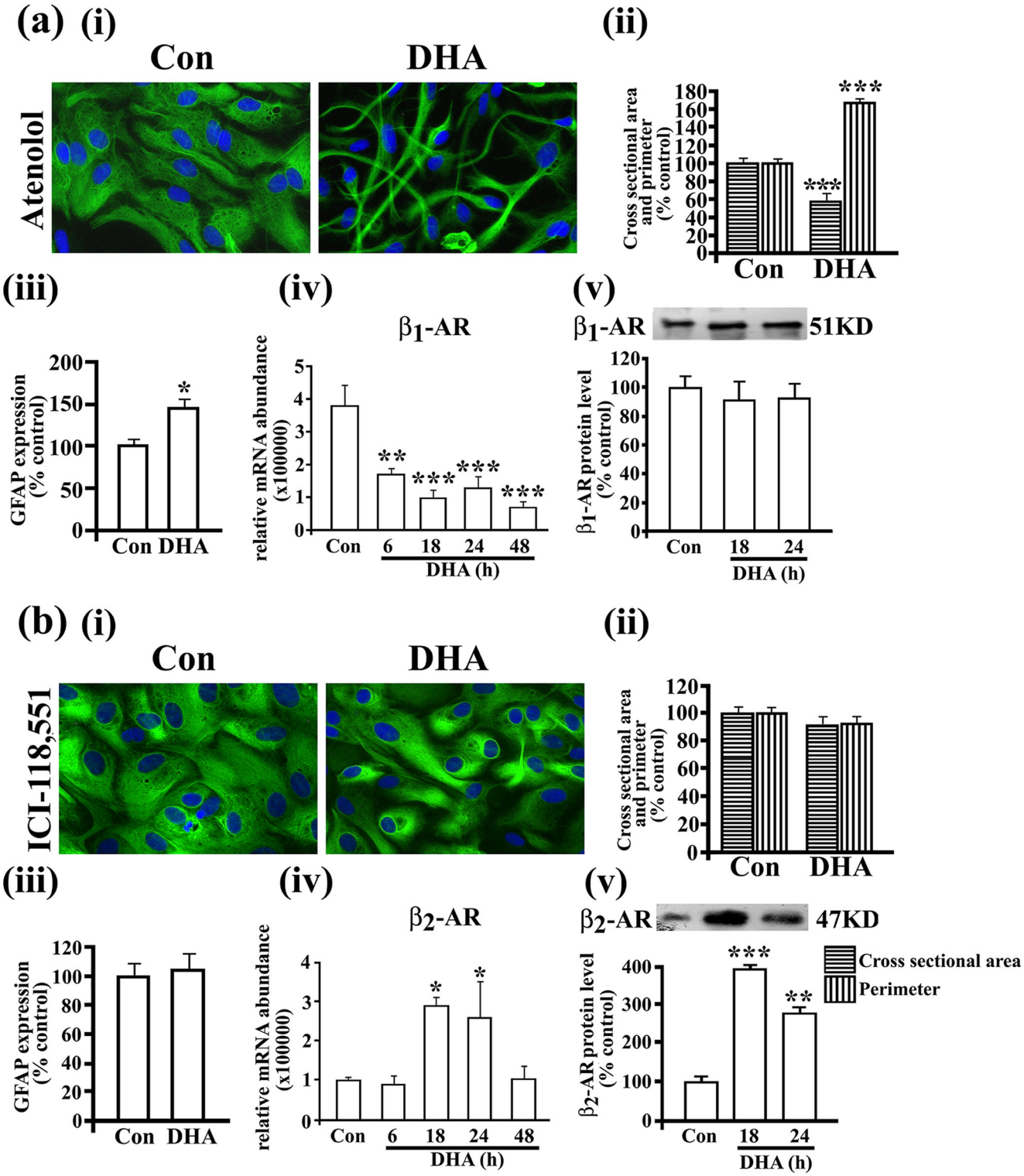

Fig. 4 Role of β-AR system on DHA-induced astrocyte differentiation. Cortical astrocytes, cultured for 10 days, were treated with either (a) atenolol (1 μM) or (b) ICI-118,551 (1 μM), prior to exposure to DHA for 48 h. (i) Immunostaining of the cells with GFAP, followed by FITC-conjugated IgG (green) and Hoechst 33342 (blue) (nucleus staining) in the presence (right panel) and absence of DHA (left panel). (ii) Graphical representation of relative changes in cross-section area, perimeter, and (iii) GFAP expression of cells after DHA treatment. (iv) Graphical representation of mRNA expression using qRT-PCR analysis of total RNA isolated from astrocytes treated with DHA for various periods (6–48 h). The relative gene expression of β_1-AR and β_2-AR normalized against the expression of GAPDH in the samples. (v) Western blot analysis of total cell lysate prepared from the astrocytes treated with DHA for the same time periods as above after subjecting to immunoprecipitation and immunoblotting with β_1-AR and β_2-AR. The level of proteins expressed during DHA treatment is expressed as a percentage of the level expressed in the absence of DHA (con). Results are expressed as mean ± SEM ($N = 4$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistical differences from control

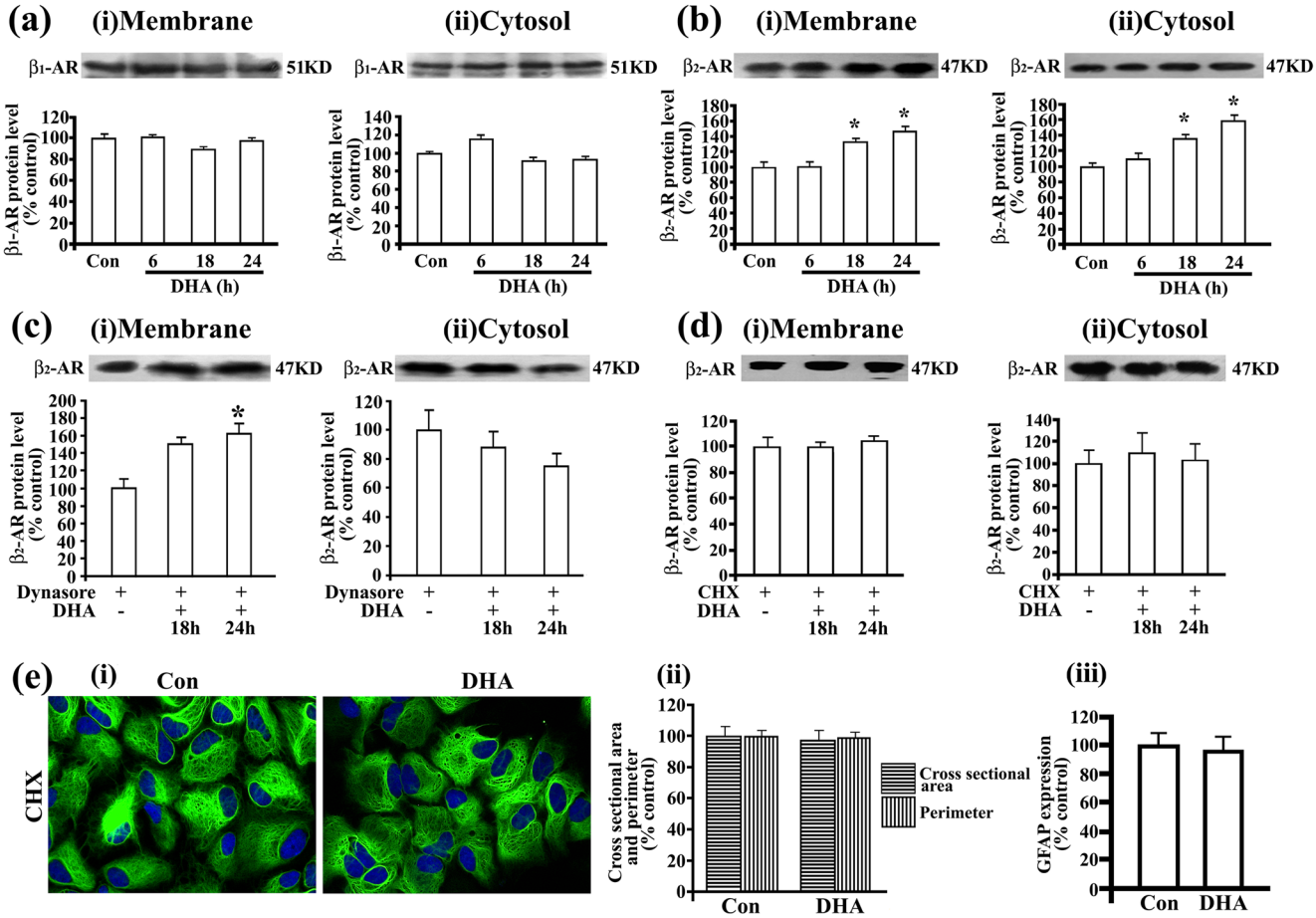

Fig. 5 Effect of DHA on β-AR isoforms of astrocytes. Membrane and cytosolic fractions of astrocyte cultures, treated with or without DHA, were subjected to immunoprecipitation and immunoblotting with (a) β_1-AR and (b) β_2-AR, as described in "Materials and Methods". (c) Cells were treated with Dynasore (80 μM) prior to DHA treatment and β_2-AR level of both (i) the membrane and (ii) cytosol was measured using similar procedure. (d) In another set of experiment cycloheximide (4 μg/ml), treatment was carried out prior to exposure of the cells to DHA, and β_2-AR levels were measured in both (i) the membrane and (ii) cytosol. The relative intensities of the β_2-AR bands indicated in the figure are represented as a percentage of the corresponding band intensities from DHA untreated controls. (ei) Morphology of the cells revealed by immunostaining by GFAP, followed by FITC-conjugated IgG (green) and Hoechst 33342 (blue) for nucleus. (eii) Change in cross-section area, perimeter, and (eiii) GFAP expression was measured and plotted. Results are expressed as mean ± SEM for $N = 4$. *$p < 0.05$ indicates statistical differences from control

DHA-Induced Differentiation Involved GPR, Upstream the Synthesis of β_2-AR

Much of the action of DHA is mediated through the endogenous GPCR receptors, GPR40, and GPR120. To test whether any of these receptors are involved in DHA-induced astrocyte differentiation, specific antagonists of these receptors were added prior to exposure of the cells to DHA. Immunocytochemical studies demonstrated that DC 260126 (antagonists of the GPR40) failed to block the DHA-induced differentiation of the 10-day-old cultured astrocytes (Fig. 8a, i–iii), whereas pretreatment with AH 7614 (antagonists of the GPR120) inhibited the differentiation of the cells induced by DHA (Fig. 8b, i–iii). However, qRT-PCR analysis showed that the mRNA levels of both GPR40 (Fig. 8aiv) and GPR120 (Fig. 8biv) receptors were unaffected during DHA treatment for the time period of 6–48 h. To confirm the involvement of GPR120, in mediating the β_2-AR transcription and astrocyte differentiation, western blot analysis of the expression level of β_2-AR in the presence of respective antagonist was carried out at 24 h, a time when DHA caused the maximum synthesis of the β_2-AR. It was observed that unlike in the DC 260126 pre-treated cells where DHA treatment continued to cause an increase in the β_2-AR level at 24 h (Fig. 8av), prior exposure of AH 7614 completely blocked the DHA-induced induction in β_2-AR level (Fig. 8bv), suggesting that GPR120 is involved in β_2-AR transcription and astrocyte differentiation.

Discussion

Our observations in the present study identify a novel signaling pathway of DHA in inducing morphological differentiation of astroglial cells. We report that DHA-induced

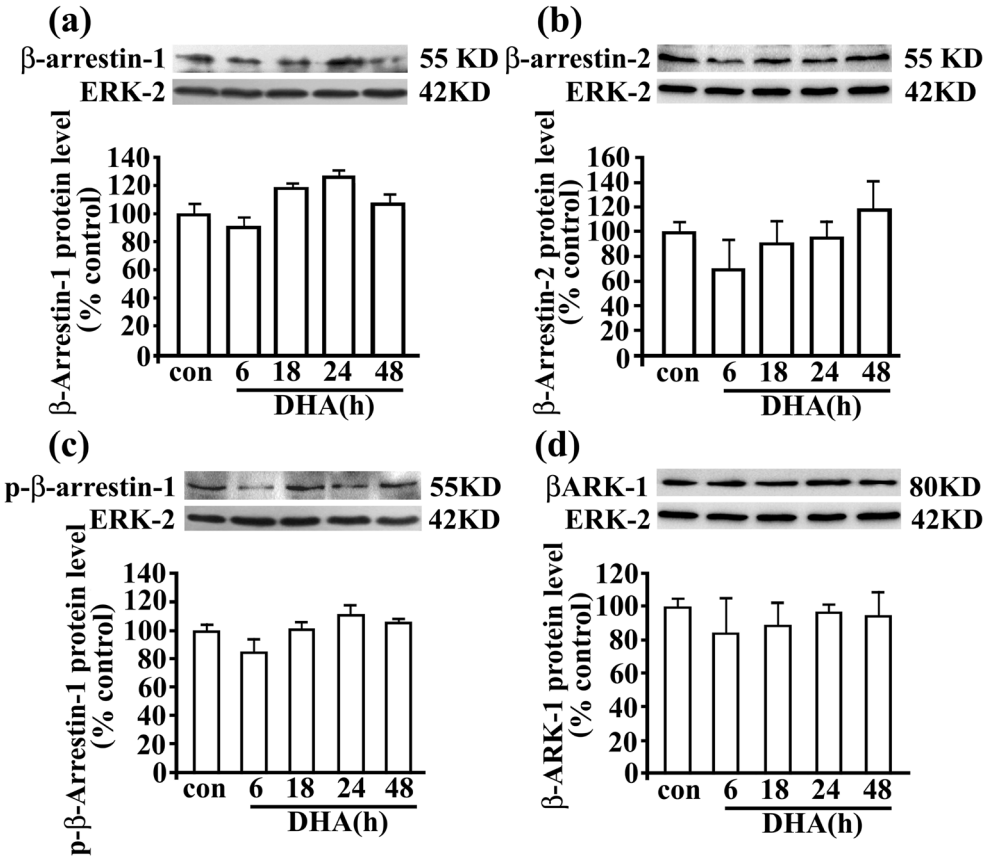

Fig. 6 Effect of DHA on the various endocytosis regulators in cultured astrocytes. Ten-day-old astrocyte cultures were exposed to DHA for the indicated time (6–48 h) and the cell extracts were subjected to western blot analysis using antibody against (a) β-arrestin-1, (b) β-arrestin-2, (c) p-β-arrestin-1, and (d) β-ARK-1 as described in "Materials and Methods". Representative western blots and internal loading control for ERK-2 are shown ($N = 3$). Densitometric analysis of protein levels is provided below the respective blots and expressed as a percentage of that expressed under TH-deficient conditions (con). The densitometric histograms represent mean ± SEM

morphological changes are mediated through β_2-AR and endocytosis of these receptors is a pre-requisite for morphological differentiation of the cells. Subsequent sustained ERK-2 activation may be responsible for driving the morphological changes in the cells.

DHA, an important lipid in the brain cell membrane, alters the membrane structural organization and dynamics and thereby modulates membrane fluidity [77, 78]. The phospholipid acyl-chain unsaturation in the lipid bilayers is the highest in DHA-containing bilayers, promoting optimal function in GPCR signaling pathways, as seen in the case of the formation of the active receptor conformation metarhodopsin II and its coupling to the retinal G-protein [79]. DHA facilitates GPCR oligomerization, thereby modulating the function of GPCR as in adenosine A2A and dopamine D2 receptor [80]. Mechanistic studies suggest the involvement of peroxisome proliferator-activated receptor [81] as well as retinoid X receptor [6] for DHA action. In addition to direct intervention in membrane structure to regulate GPCRs, DHA can also cause transcriptional regulation of some receptors like surface muscarinic receptors in cholinergic cell line NG108-15 [82] and PPARγ in human retinal pigment epithelial cells [83]. The ability of DHA to alter the expression of genes is well documented [81, 84–90]. At the level of receptors, fish oil, rich in DHA, is known to alter expression of vasopressin V1b receptor, integrin alpha 5, kainate glutamate receptor, tumor necrosis factor receptor genes, etc. [91, 92]. The present study also provides another instance where DHA induces the expression of the β_2-AR mRNA in astrocytes through transcriptional upregulation. While the present study demonstrated a significant increase in β_2-AR mRNA level at 18 and 24 h of DHA treatment that came back to basal level at 48 h, an earlier study from this laboratory [29] showed a decline in the expression of β_2-AR mRNA on exposure to DHA at 48 h. The reason for this anomaly may be due to the nature of the serum used. Unlike in the earlier work where normal serum was used, we used TH-depleted serum to rule out the influence of TH on β-ARs [64, 93, 94] and on the morphological differentiation of astrocytes [34, 47, 95].

It is observed that in addition to inducing upregulation of β_2-AR mRNA in the astrocytes, DHA exerts an inhibitory influence on the β_1-AR transcription. The 3′-UTR region of the β-AR gene contains binding sites for several protein factors like β-ARB (β-AR mRNA-binding protein), and AUF1 (AU-rich binding/degradation factor) which determine the stability and degradation of β-AR mRNA respectively. Mammalian elav-like protein HuR and the heteroribonuclear protein hnRNP A1 are important components of β-ARB, and their specific interaction with the rat β-AR isoforms based upon the 3′-UTR sequence might play a role in the agonist-mediated differential modulation of β-AR mRNA half-life [96–98]. Interestingly, DHA reduces the expression

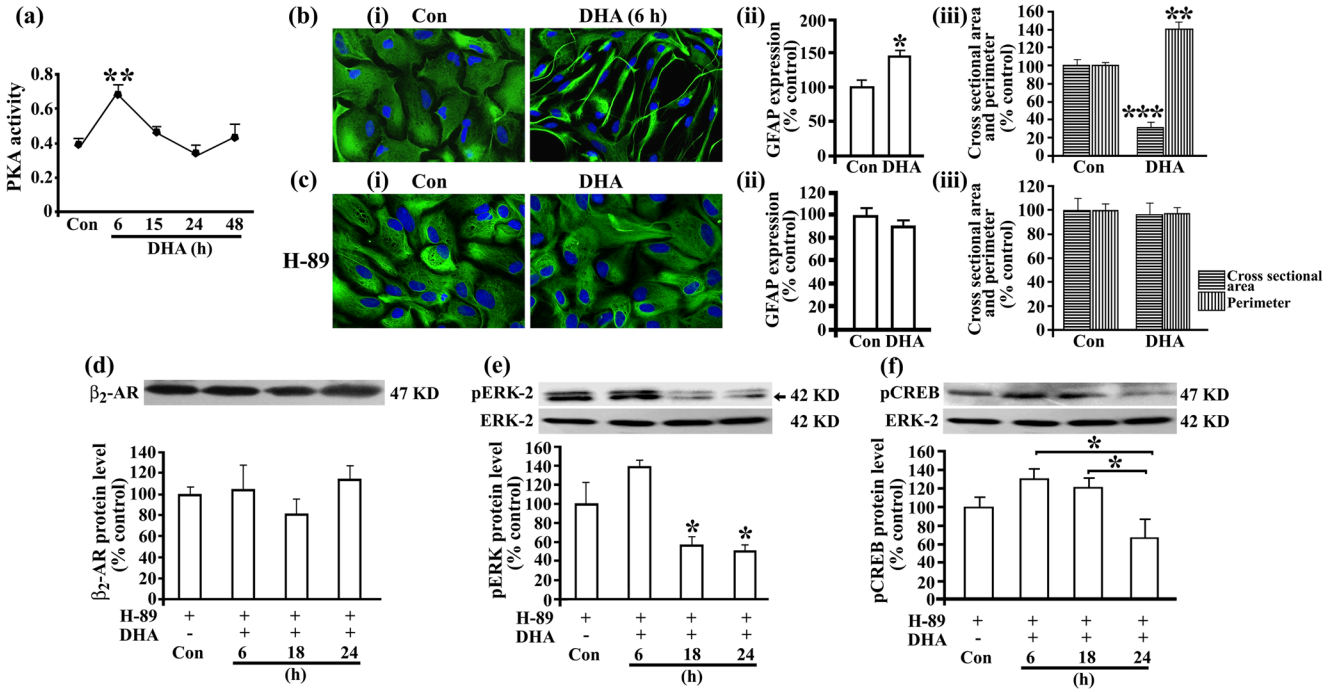

Fig. 7 Effect of DHA treatment on PKA activity in astrocytes. Ten-day-old cortical astrocyte cultures were treated with DHA for different time. (a) Cytosolic PKA activity expressed as mean ratio of activity in the absence of cAMP (intrinsic activity) divided by activity in the presence of cAMP (total activity). Values are mean ± SEM of six per group. Untreated control activity is expressed as mean ± SEM obtained from cells treated with a vehicle over the range of incubation times. (b) Cultures were exposed to the DHA for only 6 h, then replaced with DHA-deficient DMEM up to 48 h in one set of experiment, and (c) cultures supplemented with H-89 before exposure to DHA for 48 h, in another set. (i) Morphology of the cells was analyzed by immunostaining with GFAP. (ii) Cell area, perimeter, and (iii) GFAP expression were measured for each condition and represented graphically. Cell lysates obtained from H-89 and DHA treated sample were subjected to immunoprecipitation followed by immunoblotting of (d) β_2-AR, western blotting of (e) pERK-2, and (f) pCREB. The intensity of the β_2-AR bands is analyzed densitometrically expressed as a percentage of the corresponding band intensities from DHA untreated controls and represented by histogram. The histograms represent mean ± SEM independent treatments per group ($N = 3$). ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ indicated statistical difference from untreated group

of HuR in melanoma cells, thereby affecting the stability of Cox-2 mRNA [99]. Additionally, rat β_1-AR 3′-UTR shows high affinity to HuR, which mediates agonist-independent and agonist-mediated post-transcriptional degradation of the β_1-AR mRNAs [96]. There is a good possibility that DHA might regulate the expression of HuR causing reduced stability and increased degradation for β_1-AR in our study.

The importance of β_2-AR upregulation in astrocyte differentiation has been reported in a previous study [64] when transient over-expression of the β_2-AR in the astrocytes was found to be sufficient to promote the differentiation of these cells. The present study also identifies that the resultant increase in the translation of β_2-AR by DHA is critical for the differentiation process, but the regulation of β_2-AR mRNA is not well described. Early studies suggest that agonists such as isoproterenol or forskolin decreased β_2AR mRNA levels in a time-dependent manner, possibly by the activation of the cAMP system and occurs at the level of β_2AR gene transcription and not mRNA stability [98, 100]. A recent study on in vitro glioma cell model suggests that DHA also potentiates the activity of adenylate cyclase by translocating Gsα from lipid raft [101] and could be the mechanistic basis of the activation of β_2-AR mRNA, observed in the present study. The transcriptional upregulation of β_2-AR by DHA could also be attributed to the increased expression of CREB which is well documented to act as a transcription factor for the β_2-AR gene [76]. In the present study, DHA was also found to induce PKA activity as well as CREB. Interestingly, the time of activation of PKA and CREB by DHA in the astrocytes preceded the transcription of β_2-AR and could be blocked by the PKA inhibitor H-89, indicating the possibility of the involvement of PKA and CREB in the downstream transcription of β_2-AR.

The consequence of the transcriptional upregulation of β_2-AR mRNA by DHA was reflected in the increased expression of the receptor protein both in the membrane and in the cytosol which, on the other hand, remained unaltered during prior exposure to cycloheximide. GPCR like β_2-AR, upon synthesis, undergoes rapid translocation to the membrane. The increased levels of β_2-AR in the cytosol during DHA treatment could be due to the inability of the receptors to translocate to the membrane. However, increased levels of the receptors in the membrane upon DHA treatment suggests otherwise. An alternative explanation could be that increase in the membrane β_2-AR may influence increase in endocytosis of the receptors

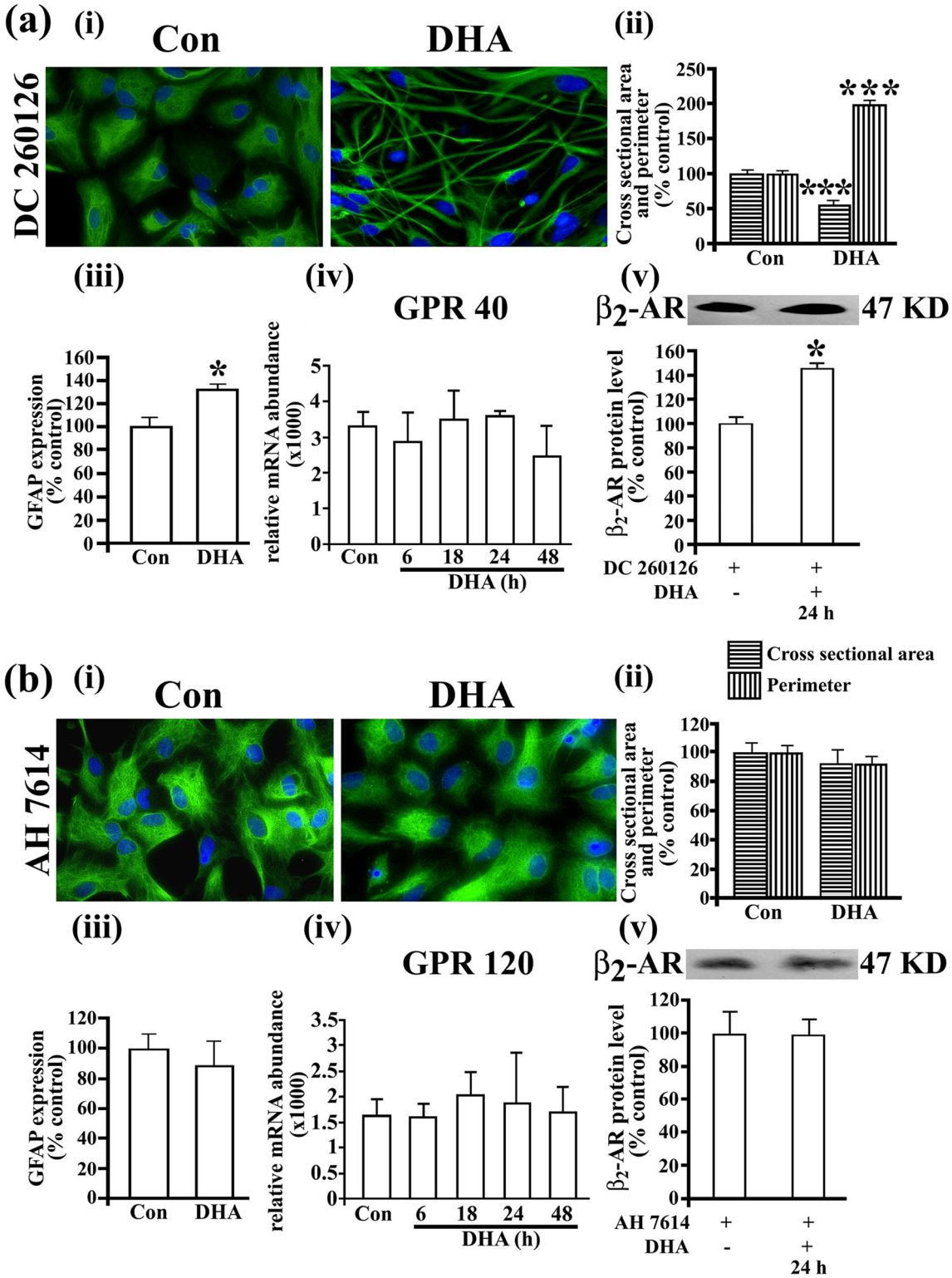

Fig. 8 Effect of DHA on GPR120 and GPR40. Ten-day-old astrocytes were treated with (a) DC 260126 (3 μM) or (b) AH 7614 (100 μM) prior to DHA treatment. (i) GFAP staining of cells followed by FITC-conjugated IgG (green) and Hoechst 33342 (blue), revealing cell morphology in the absence (Con) or presence of DHA for 48 h. (ii) Graphical representation of relative changes in the cell area, perimeter, and (iii) GFAP expression. (iv) Relative gene expression of GPR40 and GPR120 quantitated by qRT-PCR by normalizing against the expression of housekeeping gene GAPDH in the total RNA isolated from cells treated for different time (6–48 h) with DHA. (v) Quantitation of β_2-AR levels in total cell lysate of astrocytes treated with DC 260126 or AH 7614 prior to DHA treatment for 24 h by immunoprecipitation and immunoblotting with β_2-AR. The level of proteins expressed during DHA treatment is expressed as a percentage of the level of the same expressed under DHA-deficient conditions (con). Each experiment was repeated four times and results expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ indicates statistical differences from hypothyroid control

which contributes to the intracellular pool. This gets further credence from subsequent studies using the endocytic inhibitors.

We have used three inhibitors, namely, Dynasore, bafilomycin A1, and brefeldin A, to substantiate the occurrence of endosomal signaling of pERK-2 during DHA-induced morphological differentiation of the astrocytes. These inhibitors act at different steps of the endosomal pathways. Dynasore acts as a potential inhibitor of GTPase activity of dynamin protein, inhibiting the formation of coated vesicle, thereby blocking the initiation of endocytosis [102]. Bafilomycin A1, on the other hand, act further downstream to inhibit vacuolar H + ATPase, preventing endosome maturation [103], while brefeldin A, interferes with the dynamics of ER-Golgi membrane traffic to affect receptor recycling [104, 105]. Observed decline in pERK-2 level in the presence of each of the inhibitors suggests an association between endocytosis of β_2-AR and ERK activation. A number of studies suggest that late endosomal maturation involves several molecular interactions and ERK, being a regulatory factor, controls normal trafficking of cargo and its subcellular distribution. Inhibition of ERK by U0126 and PD098059 did not affect initial endocytosis process but blocked the recycling pathway by altering the morphology of the tubular endosome (recycling compartment of endosome), during the clathrin-independent endocytosis in Hela cells [106]. ERK signaling is required to maintain the membrane dynamics of the tubular endosome which is important for recycling.

It is known that during GPCR signaling, activation of ERK1/2 is triggered in two different ways, from plasma membrane and from endosome. G-protein signaling-mediated ERK1/2 activation from plasma membrane has early onset and PKA-dependent. This ERK1/2 translocates to the nucleus and elicits transcriptional activity. CREB is activated by cAMP/PKA-dependent pathway via the ERK1/2, inducing transcription of genes. During endosomal signaling, arrestin-dependent ERK1/2 activation is of late onset as it originates inside the endosome, coinciding with the waning of the G-

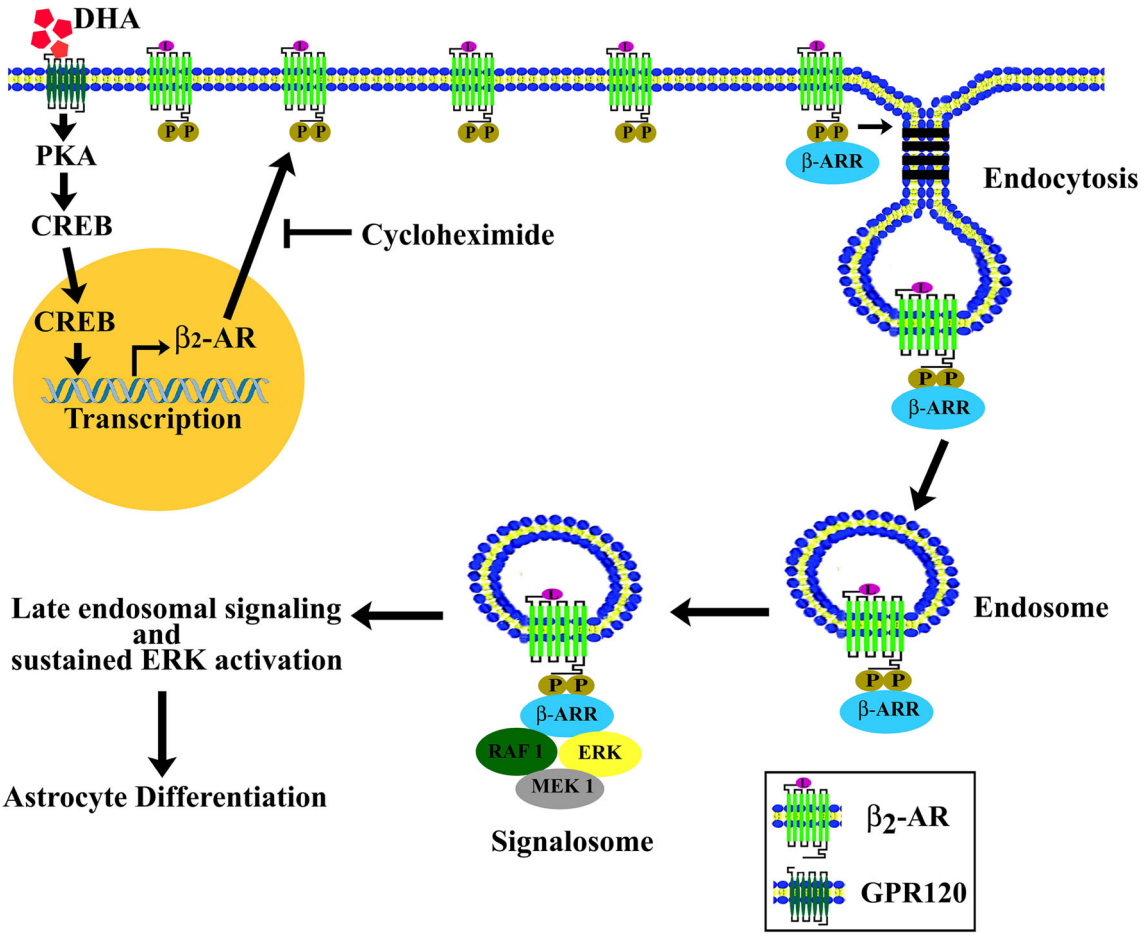

Fig. 9 Model of DHA-mediated signaling during astrocyte differentiation. DHA directly interacts with GPR120 leading to activation of PKA and downstream CREB. CREB, in turn, causes transcriptional activation of β_2-AR. Increased β_2-AR in the membrane then undergoes endocytosis. Dynamin protein, triggered by GTP binding, assembles at the necks of the invaginated coated pits and forms ring-like structure (black collar ring) causing constriction and subsequent fission of endosome. Sustained ERK activation occurred through endosomal signaling, which could promote the morphological differentiation of astrocytes

protein signals. This activated ERK1/2 is retained in the cytosol and is involved with the morphogenesis and cell survival with little transcriptional activity [62, 63]. Reports suggest that transient binding of β_2-AR-arrestin complexes dissociates upon internalization and inside the endosome, inducing arrestin-dependent activation of ERK [107]. Thus, ERK-2 activation during exposure to DHA occurs by upstream PKA and CREB which in turn facilitates transcription of β_2-AR as well as at the downstream of endocytosis of β_2-AR.

Such sustained induction of ERK is generally a delayed event [107]. Our previous studies have shown that TH, as well as isoproterenol, mediated astrocyte differentiation that is associated with a sustained induction of ERK only after 18 h of the hormone treatment [41, 47]. In the present study, DHA caused a sustained induction of ERK from 6 h onwards. Interestingly, the magnitude of this early induction was significantly more than in the later phase and coincided with the peak increase in PKA activity during DHA treatment. Moreover, it was also sensitive to the PKA inhibitor H-89. Crosstalk between GPCR and ERK is reported in the literature [108, 109], and G-protein-mediated ERK is found to translocate to the nucleus and is involved in transcriptional regulation of several astrocyte differentiation marker genes [57]. Moreover, during DHA-mediated signaling, pERK formation mainly involves PKA pathway [110], suggesting that the initial increase in PKA activity during exposure to DHA could contribute toward the early surge in pERK levels.

On the contrary, endocytosis of β_2-AR and other GPCRs is generally driven by β-arrestins and the resultant activation of ERK has been found to be mainly regulated by β-arrestin in the endosome [63, 108, 111]. However, in the present study, no alteration in the expression and activity of the isoforms of β-arrestins was observed during exposure to DHA. Possibly, the increase in the synthesis of β_2-AR by DHA contributed to the resultant increase in endocytosis of the β_2-AR with the active participation of the existing levels of β-arrestins present in the cell.

Overall, the present study presents a mechanistic view of the morphological differentiation of astrocytes induced by DHA (Fig. 9). Stellation of astrocytes can be quantified by CSF calculation, where perfectly round cells have a shape factor close to 1, whereas more elongated cells (with long process) exhibit lower values, approaching 0 [112]. The changes in CSF under the different treatment conditions were presented in Table 2. The significant decrease in CSF is observed in cells in the presence of DHA, atenolol; DC 260126 also support the idea that DHA induces stellation and the signaling is unrelated to β_1-AR and GPR40. Rather, DHA directly interacts with GPR120 and causes activation of PKA and CREB-mediated transcription of another GPCR, β_2-AR. The induction of β_2-AR, both at the protein and mRNA levels, results in increased turnover of the β_2-AR. This, in turn, facilitates increased endocytosis of the β_2-AR. Eventually, endosomal signaling by β_2-AR is triggered leading to the sustained activation of pERK-2, which drives the process of differentiation in the astrocytes.

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Compliance with Ethical Standards

Animal experimentation was approved by the institutional animal ethics committee appointed by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) of the animal welfare division under the Ministry of Environment and Forest, Government of India (Registration no. 147/CPCSEA).

Conflict of Interest The authors declare that they have no conflict of interest.

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