



Prostaglandin E2 facilitates subcellular translocation of the EP4 receptor in neuroectodermal NE-4C stem cells



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ABSTRACT

Prostaglandin E2 (PGE₂) is a lipid mediator released from the phospholipid membranes that mediates important physiological functions in the nervous system via activation of four EP receptors (EP1–4). There is growing evidence for the important role of the PGE₂/EP4 signaling in the nervous system. Previous studies in our lab show that the expression of the EP4 receptor is significantly higher during the neurogenesis period in the mouse. We also showed that in mouse neuroblastoma cells, the PGE₂/EP4 receptor signaling pathway plays a role in regulation of intracellular calcium via a phosphoinositide 3-kinase (PI3K)-dependent mechanism. Recent research indicates that the functional importance of the EP4 receptor depends on its subcellular localization. PGE₂-induced EP4 externalization to the plasma membrane of primary sensory neurons has been shown to play a role in the pain pathway. In the present study, we detected a novel PGE₂-dependent subcellular trafficking of the EP4 receptor in neuroectodermal (NE-4C) stem cells and differentiated NE-4C neuronal cells. We show that PGE₂ induces EP4 externalization from the Golgi apparatus to the plasma membrane in NE-4C stem cells. We also show that the EP4 receptors translocate to growth cones of differentiating NE-4C neuronal cells and that a higher level of PGE₂ enhances its growth cone localization. These results demonstrate that the EP4 receptor relocation to the plasma membrane and growth cones in NE-4C cells is PGE₂ dependent. Thus, the functional role of the PGE₂/EP4 pathway in the developing nervous system may depend on the subcellular localization of the EP4 receptor.

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1. Introduction

Prostaglandin E2 (PGE₂) is a bioactive lipid derived from plasma membrane phospholipids, through enzymatic metabolism of arachidonic acid by cyclooxygenases -1 and -2 (COX -1,-2) and prostaglandin synthase [1]. PGE₂ mediates biosynthetic pathways that regulate biological functions such as sleep, fever, inflammation, and pain [2]. PGE₂ also plays a critical role in the proper development of the nervous system. It induces differentiation of neuronal cells [3] and plays a regulatory role in membrane excitability and synaptic transmission in neurons [4]. PGE₂ can also increase dendritic length and alter neuronal firing activity in the brain [5].

PGE₂ exerts its physiological function through its four cell surface G protein-coupled receptors (GPCRs) designated EP (E-Prostanoid) 1–4, with different affinities [1,6,7]. Activation of EP1

receptor is associated with an increase of intracellular calcium [Ca²⁺]_i, mediated by phospholipase C and inositol 1,4,5-triphosphate (IP3) [7,8]. EP2 and EP4 are coupled to the stimulatory G_s protein and cause an increase in cAMP through activation of adenylate cyclase [9], which in turn activates protein kinase A (PKA) and mediates phosphorylation of cAMP response element binding the protein (CREB) transcription factor [10]. Activation of a specific EP3 isoform, can both decrease and increase cyclic AMP (cAMP) and IP3. It is also shown that the EP4 receptor signaling can operate via G_i proteins- phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways [11,12].

Interest in the PGE₂/EP4 pathway is increasing given its diverse capability of regulating central nervous system activity [13]. EP4 has a protective function by reducing cerebral injury and improving functional outcome after stroke [14], and in suppressing brain inflammation [15]. The EP4 receptor has been suggested to contribute to PGE₂-induced changes in body temperature [16]. Furthermore, EP4 activation can decrease the level of amyloid-beta in the brain and improve behavioral performance in a murine model of Alzheimer's disease [17]. The EP4 receptor along with EP3C (EP3γ) mediates PGE₂-induced sensitization of sensory

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neurons [18]. PGE₂-prolonged sensitization of nociceptive dorsal root ganglion neurons may also contribute to the transition from acute to chronic pain by facilitating EP4 receptor synthesis and anterograde axonal trafficking [19]. We have previously found that the PGE₂/EP4 pathway plays an inhibitory role in regulating the intracellular calcium homeostasis in mouse neuroblastoma (Neuro-2a) cells via PI3K mechanism [20]. Expression of the EP4 receptor is higher during early neurogenesis as compared to later embryonic stages in mouse embryos suggesting its importance in the developing nervous system [21].

Recent research shows that the subcellular trafficking of the EP4 receptor may have functional implications. It has been shown that PGE₂-induced EP4 externalization to the plasma membrane in dorsal root ganglion neurons is important for the inflammatory pain response [22]. The goal of this study was to determine whether PGE₂ can also induce EP4 receptor trafficking in neuroectodermal (NE-4C) stem cells used as an experimental model system for early neuronal development. We show that PGE₂ causes translocation of the EP4 receptor from its normal location in the Golgi apparatus [23] to the plasma membrane in undifferentiated NE-4C stem cells. This was confirmed with a specific EP4 receptor agonist. We also show for the first time that PGE₂ can enhance trafficking of the EP4 receptor to growth cones of differentiated neuronal NE-4C cells. This study shows that PGE₂ can influence the subcellular localization of the EP4 receptor in neuronal stem cells and differentiated neuronal cells.

2. Methods

2.1. Cell cultures

Mouse neuroectodermal (NE-4C) stem cell line was obtained from American Tissue Culture Collection (ATCC) and grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1X penicillin-streptomycin mixture (Invitrogen). Cells were maintained in an incubator containing 5% CO₂ at 95% humidity at 37 °C. Cells were plated on 0.01% poly-L-lysine (Sigma) coated 100 mm culture plate (BD Falcon) and subcultured at a 1:10 ratio. Supplemented MEM was changed every 2 days. NE-4C cells were seeded onto culture plates containing poly-L-lysine and incubated overnight at 37 °C before treatment.

2.2. Differentiation of NE-4C stem cells into neurons

Differentiation of NE-4C cells was induced on day 0 using Neurobasal media (NBM; supplemented with L-glutamate, 1 × Pen Strep, and 1 × B-27; Invitrogen) in poly-L-lysine (Sigma, MW 70,000–150,000 kDa) pre-coated 100 mm culture plates. By day 6, neurospheres (clusters of neural stem cells) were dissociated, seeded onto 35 mm culture plates containing poly-L-lysine coated coverslips and grown until day 12. Supplemented differentiating media was replaced every 2 days. Pou Class 5 homeobox 1 (*Pou5f1*) and Mouse BIII tubulin (*Tubb3*) were used on day 12 as stem cell and late neuronal cell markers to confirm differentiation using PCR. PCR primers are as follows: *Pou5f1* forward 5'-CTGGCTAAGCTTCCAAGGGC-3' and reverse 5'-CCAGGGTCTCCGATTTGCAT-3' *Tubb3* 5'-AGCAGCTACTCTCGTGGAGTG-3' and reverse 5'-GGGCTTCCGATTCCTCGTCA-3'. As compared to day 0, which showed presence of the *Pou5f1* marker, we confirmed that on day 12 only the *Tubb3* marker was detected (data not shown).

2.3. Cell culture treatments

To explore the time course of PGE₂-induced EP4 externalization, we subjected NE-4C cells to 10 μM PGE₂ (Sigma) for 1, 3 and 24 h (hours). The abundance of EP4 at the plasma membrane appeared after 3 h, thus we selected the 3 h time for the following experiments. We also subjected NE-4C cells to 1 and 10 μM concentration of PGE₂ for 3 h (no effect seen by 1 μM). Controls contained a corresponding concentration of the PGE₂ organic solvent DMSO (dimethyl sulfoxide).

PGE₂-dependent EP4 externalization was confirmed using EP4 agonist (CAY10580; Cayman Chemicals; 10, 50, 100 μM) for 3 h, with representative figures presented. CAY10580 is commonly used because it is highly specific (60–85 times) to the EP4 receptor compared to the other EP receptors [24]. Specificity of this externalization was confirmed through use of EP4 antagonist (AH23848; Cayman Chemicals; 100 μM) alone. To ensure sufficient EP4 receptor blocking prior to co-treatment with the agonist or PGE₂, a pre-treatment (1 h) was applied followed by co-treatment (3 h) with CAY10580 (100 μM) and PGE₂ (10 μM). PGE₂-induced EP4 translocation was also performed in NE-4C cells differentiated into neurons after 12 days. PGE₂ (1 and 10 μM) was applied to differentiated cells for 3 and 24 h, and compared to the untreated control group (DMSO vehicle). Treatment concentrations of PGE₂, CAY10580, and AH23848 tested in this study are within a concentration range widely used in various neural cell types [21,22,25–27].

2.4. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (4.3 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄) for 20 min at 4 °C and rinsed twice with PBS. Cells were then permeabilized with 0.3% Triton X-100 and 2% Normal Goat Serum (NGS) in PBS for 20 min, followed by incubation of primary antibodies in TBS-T (TBS-Tween 20, 0.05%) and 2% NGS.

Cellular localization of the EP4 receptor in NE-4C cells was determined as we previously described [23]. In brief, incubation with anti-rabbit EP4 primary antibody (1:60; Santa Cruz Biotechnologies) along with monoclonal anti-mouse PMCA1 plasma membrane marker (1:500; Abcam), at room temperature for 2 h. Following primary antibody incubation, cells were washed three times with PBS for 5 min and incubated with secondary antibodies in TBS-T (TBS-Tween 20, 0.05%) and 2% NGS for 1 h at room temperature in the dark.

Secondary antibodies used were anti-rabbit fluorescein isothiocyanate (FITC) (1:100; Jackson ImmunoResearch Laboratories) and anti-mouse Texas Red (1:200; Jackson ImmunoResearch Laboratories). Cells were then washed three times with PBS for 5 min, followed by a 10 min incubation of 4',6-diamidino-2-phenylindole (DAPI) (1:10,000; Molecular Probes) at room temperature in the dark. Cells were washed three times with PBS for 5 min and coverslips were mounted on glass microscope slides with anti-fade mounting media (Invitrogen). The staining was visualized and captured using an Olympus Fluoview 300 Confocal Laser Scanning Microscope. Secondary antibodies were used without primary antibodies to serve as a control for specificity (not shown). Control staining completed with the absence of respective primary antibody revealed that each antibody was specific to the target (not shown). We also previously tested the specificity of the same EP4 primary antibody via immunocytochemistry and western blot analysis [21,23].

2.5. Immunocytochemistry quantification

NIS Elements software (Nikon) was used to measure the signal intensity of EP4 receptor at the plasma membrane in undifferentiated NE-4C stem cells. A line of 3pt width was drawn around the plasma membrane (perimeter of the cell) using the PMCA1 marker to define the perimeter. Once the plasma membrane was outlined, the region of interest (ROI) was overlaid on the EP4 image in the same location, and then the mean signal intensity was measured. The mean signal intensity accounts for the area of the ROI (line; length \times width) overlaying the plasma membrane. The EP4 signal intensity is presented as a ratio in comparison to the control. Growth cone signal intensity of fluorescence was measured using the same technique with ImageJ software [28]. EP4 receptor intensity in the plasma membrane and at the growth cone was taken as a mean for each treatment and compared to the mean of the control (DMSO, vehicle), and presented as a ratio value in comparison to the control. Background

signals were subtracted from the signal intensity measurements for each cell and growth cone. Treatment groups were compared to the untreated group and was considered statistically significant at $p < 0.05$ by ANOVA and/or a student's *t*-test.

3. Results

3.1. PGE₂ induces EP4 externalization to the plasma membrane in undifferentiated NE-4C stem cells

We previously established that undifferentiated neuroectodermal NE-4C stem cells express all EP1-4 receptors and that the EP4 receptor is normally localized in the Golgi apparatus [23]. To determine if PGE₂ can induce EP4 cell surface externalization in NE-4C cells, undifferentiated NE-4C cells were treated with 1 and 10 μ M of PGE₂. Similar to untreated NE-4C cells, the 1 μ M and 10 μ M PGE₂-treated cells show EP4 present in the Golgi apparatus (only 10 μ M PGE₂ is

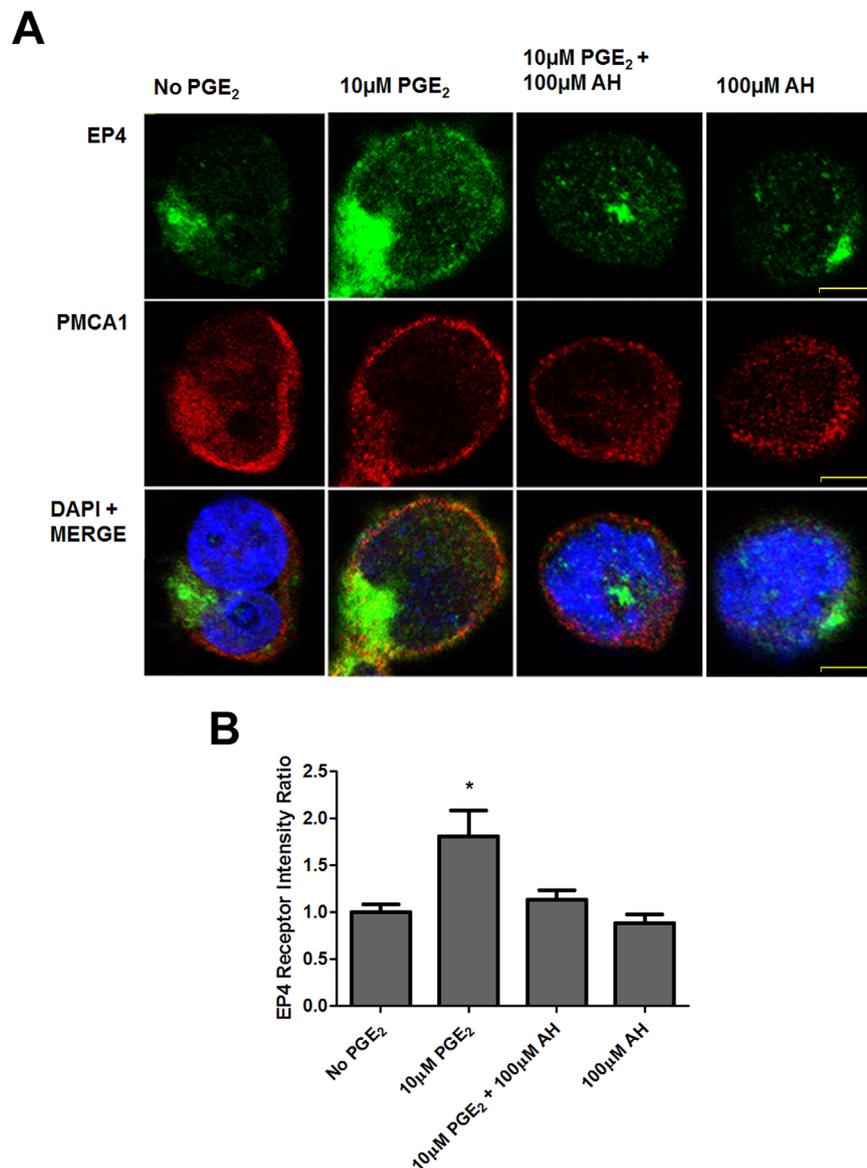


Fig. 1. PGE₂-induced EP4 localization at the plasma membrane in undifferentiated NE-4C stem cells. A. Immunocytochemistry visualization of EP4 receptor. The EP4 receptor (Anti-EP4; top panel); plasma membrane marker (anti-PMCA1; middle panel); merged images (the nucleus marker DAPI; lower panel). EP4 localizes to the Golgi apparatus in no PGE₂ control and at the plasma membrane with 10 μ M PGE₂. The plasma membrane localization was blocked by the EP4 antagonist (10 μ M PGE₂ + 100 μ M AH), and there was no effect with antagonist alone (100 μ M AH). The scale bar represents 10 μ m. B. Quantification of immunofluorescent EP4 receptor localization at the plasma membrane depicted as a ratio value in comparison to no PGE₂ group (set at 1.0); 10 μ M PGE₂ significantly increased the EP4 at the plasma membrane ($p < 0.05$). PGE₂=prostaglandin E2.

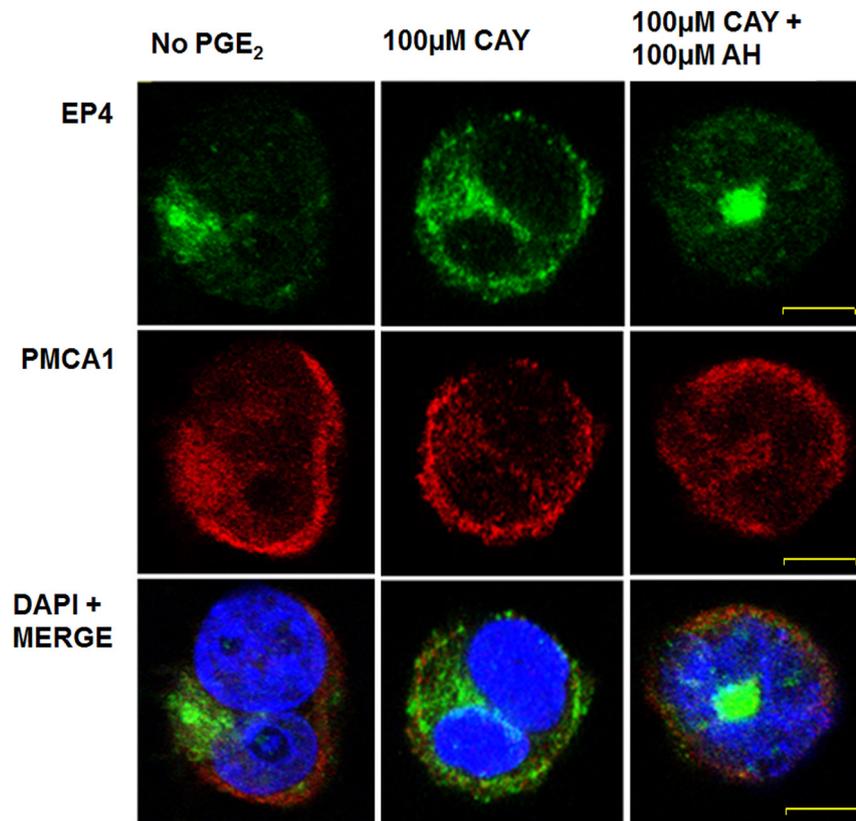
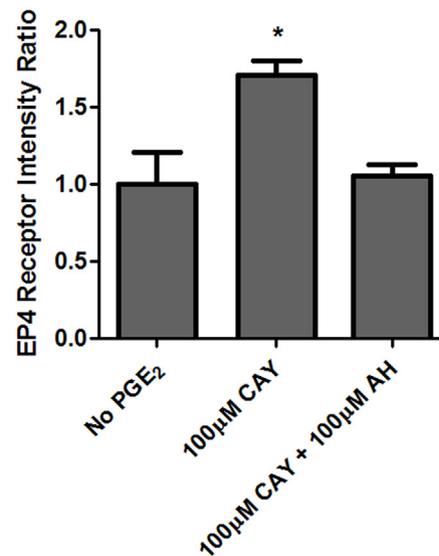
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Fig. 2. Agonist-induced EP4 localization at the plasma membrane in undifferentiated NE-4C stem cells. A. Immunocytochemistry visualization of EP4 receptor. The EP4 receptor (Anti-EP4; *top panel*); plasma membrane marker (anti-PMCA1; *middle panel*); merged images (the nucleus marker DAPI; *lower panel*). EP4 receptor localization at the plasma membrane with EP4 agonist (100 μM CAY) was blocked with EP4 antagonist (100 μM CAY + 100 μM AH). The scale bar represents 10 μm. B. Quantification of immunofluorescent EP4 receptor localization at the plasma membrane depicted as a ratio value in comparison to no PGE₂ group (set at 1.0); 100 μM CAY significantly increased the EP4 at the plasma membrane ($p < 0.05$).

shown in Fig. 1(A)). However, with 10 μM PGE₂ only, EP4 receptor colocalized in the peripheral region with the PMCA1 (plasma membrane Ca²⁺-ATPase) marker, suggesting dose-dependent PGE₂-induced EP4

translocation toward the plasma membrane (Fig. 1(A)). The EP4 plasma membrane externalization in NE-4C cells became apparent after 3 h exposure to 10 μM PGE₂.

The observed PGE₂-induced EP4 externalization to the plasma membrane was inhibited by a selective EP4 antagonist, AH23848. AH23848 alone had no effect on the EP4 trafficking (Fig. 1(A)). This indicates that the trafficking likely occurred through the EP4 receptor. We verified the increased abundance of EP4 receptor at the plasma membrane through immunofluorescence quantification (Fig. 1(B)). Immunofluorescence intensity ratios in cells treated with PGE₂ and with the addition of the EP4 antagonist (AH23848) were calculated by comparing the EP4 staining intensity in the cell membrane of each corresponding treatment condition to the control condition without PGE₂. These results show a novel PGE₂-dependent subcellular translocation of the EP4 receptor in NE-4C stem cells.

3.2. EP4 receptor agonist induces EP4 externalization to the plasma membrane in undifferentiated NE-4C stem cells

The PGE₂-induced EP4 externalization in undifferentiated NE-4C cells was confirmed with a selective EP4 agonist (CAY10580). We used 10, 50 and 100 μM treatment with CAY10580 for 3 h [22]. Similar to PGE₂, the CAY10580 also induced EP4 externalization to the plasma membrane. All CAY10580 concentrations tested show the Golgi expression whereas the 100 μM significantly increased the EP4 externalization to the plasma membrane (Fig. 2(A)). AH23848 blocked CAY10580-induced EP4 externalization (Fig. 2(A)), which was confirmed through immunofluorescence quantification via intensity ratios (Fig. 2(B)). Immunofluorescence intensity ratios of EP4 staining were calculated by comparing the treatment conditions to the control without PGE₂. Overall, these results suggest that the EP4 receptor subcellular re-location from the Golgi apparatus to the plasma membrane is regulated through the EP4 signaling pathway.

3.3. PGE₂ dependent EP4 receptor localization in differentiated NE-4C neuronal cells

Given the increasingly important role of EP4 in the developing nervous system, we also assessed the localization of the EP4 receptor in differentiated NE-4C neuronal cells. Similar to NE-4C stem cells, we only observed typical Golgi localization in differentiated NE-4C cells (Fig. 3) after exposure to PGE₂ with no apparent trafficking to the plasma membrane. Interestingly, we show for the first time that the endogenous EP4 receptor was also present in the growth cones of untreated differentiated NE-4C cells (Fig. 4(A)). Moreover, exposure to 1 and 10 μM concentrations of PGE₂ resulted in significantly increased localization of EP4 in the growth cones after 3 h that remained unchanged for the duration

of 24 h (Fig. 4(A) and (B)). There was no significant difference between the 1 and 10 μM PGE₂ treatments at 3 or 24 h.

4. Discussion

The results of this study show that 10 μM PGE₂ induces EP4 externalization from its previously characterized localization in the Golgi apparatus [23] to the plasma membrane in undifferentiated NE-4C stem cells. 1 μM PGE₂ did not have a significant effect on undifferentiated NE-4C stem cells, indicating that EP4-translocation may be PGE₂ concentration-dependent. Interestingly, we also found that in differentiated NE-4C neuronal cells, 1 or 10 μM PGE₂ enhanced EP4 trafficking to the growth cones. The observed subcellular translocation of the EP4 receptor from its normal location in the Golgi apparatus to the plasma membrane and growth cones indicates that it may play an important role in early function of neuronal cells.

The functional importance of PGE₂-induced subcellular localization of the EP4 receptor in the developing nervous system is still largely unknown. Whether EP4 signaling in various subcellular compartments of NE-4C cells is regulated by G_s and/or G_i proteins still needs further investigation. PGE₂-mediated EP4 activation of either G_s or G_i may result in increased or decreased cAMP-PKA signaling respectively [29]. EP4 activity may also stimulate cAMP-independent signaling through PI3K activation [29]. However, there is growing evidence for the functional importance of the PGE₂/EP4 signaling in the developing nervous system. The PGE₂/EP4 pathway has been suggested to play a role in the transition from acute to chronic pain in nociceptive dorsal root ganglion neurons [19]. St. Jacques and Ma found that PGE₂-prolonged sensitization of neurons facilitated the synthesis and anterograde axonal trafficking of EP4 receptors [19]. Our previous study shows that EP4 is involved in PGE₂-dependent regulation of intracellular calcium level through a novel PI3K inhibitory mechanism and it also reduces neurite lengths in differentiated Neuro-2a cells [20]. This is interesting because calcium ions are key mediators to multiple cellular processes in early neuronal development. For example, in neuronal growth cones calcium contributes to axonal growth and guidance, which must be strictly regulated during neuronal development [30–32]. Interestingly, we also showed that the level of the EP4 receptor is higher in the mouse embryo (embryonic stage 7, 11 and 15) as compared to the later stage E17 [21], indicating its important role in early development.

PGE₂-EP4 activation has been found to attenuate the activation of microglia and to prevent lipid peroxidation and pro-inflammatory gene expression in a murine model of lipopolysaccharide (LPS)-induced

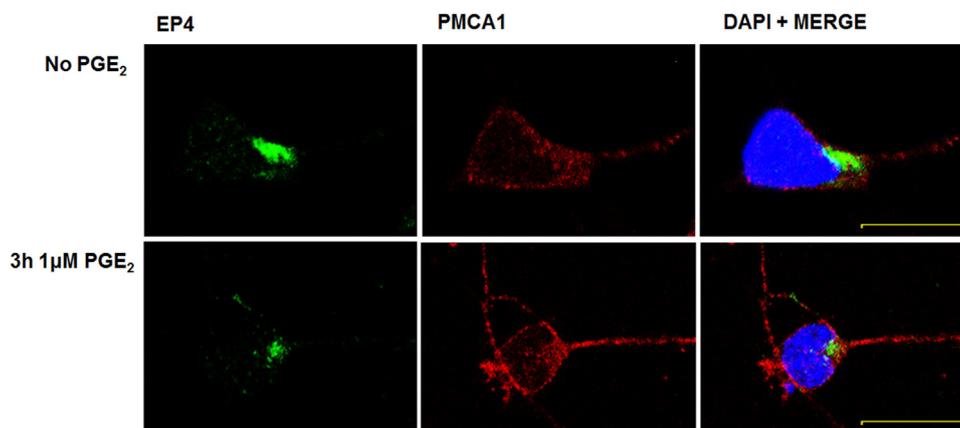


Fig. 3. EP4 receptor localization in differentiated NE-4C cells. The EP4 receptor (Anti-EP4; left panel); plasma membrane marker (anti-PMCA1; middle panel); merge images (the nucleus marker DAPI; right panel). EP4 receptor is localized in the Golgi apparatus. Scale bar represents 10 μm. PGE₂=prostaglandin E2.

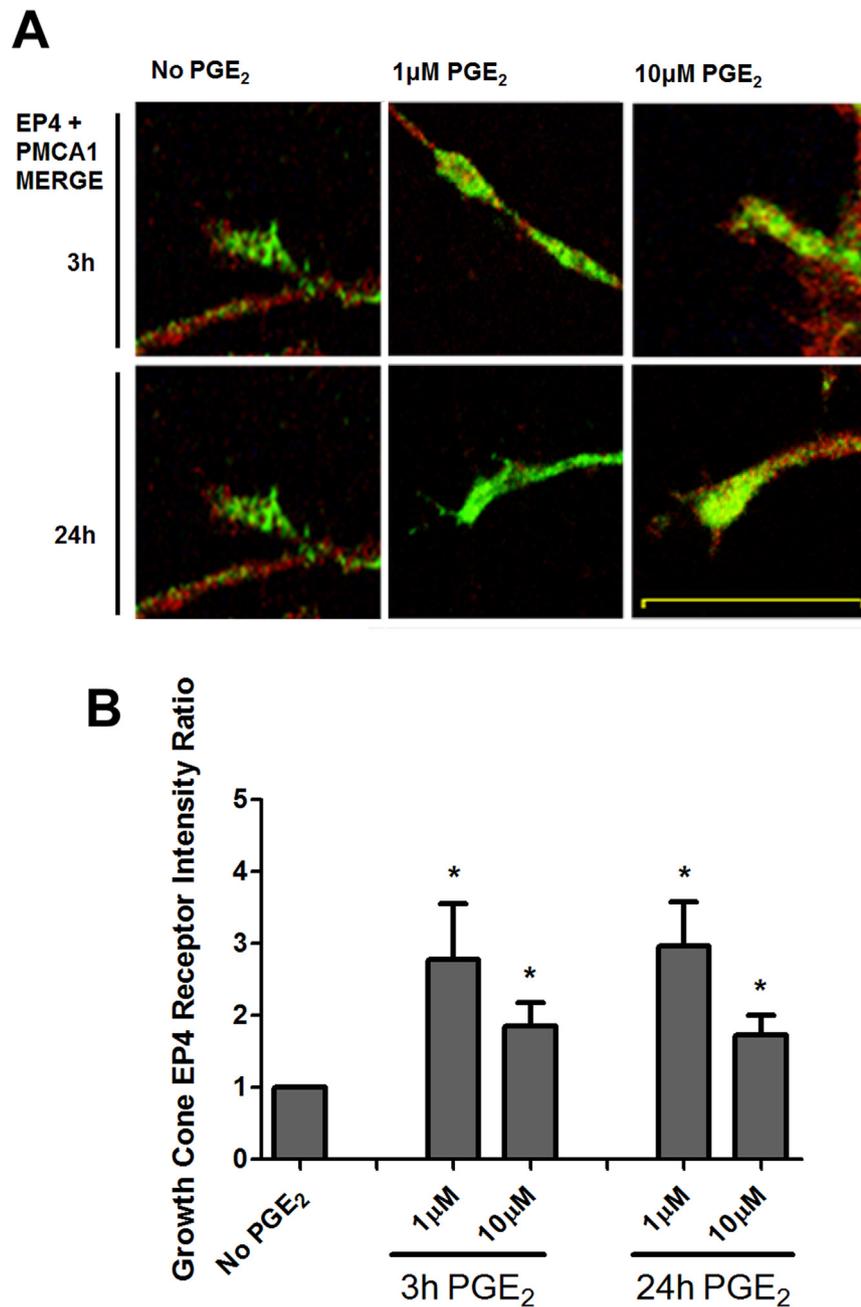


Fig. 4. EP4 Receptor localization in the growth cones of differentiated NE-4C cells. A. The EP4 receptor (*Anti-EP4*) merged image with plasma membrane marker (*Anti-PMCA1*). 1 μ M and 10 μ M PGE₂ treatment for 3 h (*top row*), 24 h (*bottom row*). Scale bar represents 5 μ m. B. Quantification of immunofluorescent EP4 receptor localization in the growth cones using only the green fluorescent channel depicted as a ratio value in comparison to *no PGE₂* group (set at 1.0); Both 1 μ M and 10 μ M PGE₂ significantly increased the EP4 in the growth cones after 3 and 24 h ($p < 0.05$). PGE₂=prostaglandin E2.

brain inflammation [15]. Moreover, PGE₂/EP4 signaling has elicited a protective function in reducing injury and improving functional recovery after stroke via dual and independent cell-specific mechanisms of neuroprotection and enhanced vascular perfusion [14]. In contrast, genetic and pharmacologic inhibition of EP4 receptor, via EP4 deficiency or antagonist respectively, in a murine mouse model of Alzheimer's disease decreased amyloid- β levels in the brain and improved the behavioral performance of the animals [17]. EP4 receptors are the most widely expressed PGE₂ receptors in the body [13], and the various biological effects observed due to PGE₂ signaling via EP4 may be mediated by the externalization of EP4 to the plasma membrane and growth cones. Although the observed trafficking of the EP4 receptor to the plasma membrane induced by PGE₂ and the EP4-specific agonist is compelling, the potential contribution of remaining EP receptors needs to be considered as well.

In summary, these results confirm that the EP4 externalization from the Golgi apparatus to the plasma membrane in NE-4C stem cells is PGE₂-induced. Furthermore, this study provides the first evidence that PGE₂ can also enhance the growth cone localization of the EP4 receptor in differentiating NE-4C neuronal cells. Our results show that the important role of the PGE₂/EP4 pathway in the developing nervous system may depend on the subcellular localization of the EP4 receptor.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.06.001>.

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