Role of Glycine Receptor α3 in the Phosphorylation of Extracellular Signal-Regulated Kinase by Prostaglandin E_2

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Research

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Abstract

Background: Glycine receptors (GlyRs) play a key role in the processing of inflammatory pain. We used Adeno-associated virus (AAV) for GlyRα1/3 gene transfer in F11 neuron cells, and investigated the effects and roles of pAAV-GlyRα1/3 on cell cytotoxicity and the prostaglandin E_2 (PGE_2)-mediated inflammatory response.

Methods: pAAV-GlyRα1 and pAAV-GlyRα3 recombinant vectors were constructed, and cell viability was measured following pAAV-GlyRα1/3 transfection. The activation of mitogen-activated protein kinase (MAPK) inflammatory signaling and neuronal injury marker activating transcription factor 3 (ATF-3) were evaluated by western blotting; the level of cytokine expression was measured by ELISA.

Results: We found that pAAV/pAAV-GlyRα1/3 transfection slightly, but not significantly, increased cell viability and induced extracellular signal-regulated kinase (ERK1/2) phosphorylation and ATF-3 activation. However, the transfection reagent lipofectamine significantly increased cell death and induced ERK1/2 phosphorylation and ATF-3 activation. More importantly, the PGE_2-induced ERK1/2 phosphorylation in F11 cells was repressed by the expression of pAAV-GlyRα3 and administration of an EP_2 inhibitor (PF-04418948), GlyRαs antagonist (strychnine), and protein kinase C inhibitor (G06983).

Conclusions: PGE_2-induced ERK1/2 phosphorylation can be modulated by GlyRα3. In addition, no changes in the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, or IL-6 of the F11 cells were observed 6 hours after lipopolysaccharide (LPS) or complete Freund's adjuvant (CFA) treatment. These data suggest that delivering pAAV-GlyRα3 into neuron cells should be safe for downregulating PGE_2-induced ERK1/2 phosphorylation and inflammation.

Background

Glycine receptors (GlyRs) are anion-permeable pentameric ligand-gated ion channels that belong to the Cys-loop superfamily of ligand-gated ion channels.[1] Previous studies found GlyRs in the brain, spinal cord, and dorsal root ganglion (DRG) of mammals.[2, 3] They play critical roles in the mammalian central nervous system, including in motor coordination,[4] hyperekplexia,[5] epilepsy,[6] autism,[7] and pain signaling.[8] There is also evidence that part of the spinal component of inflammatory hyperalgesia is due to diminished glycinergic inhibition caused by the phosphorylation and inhibition of GlyRα3.[9–12] Prostaglandin E_2 (PGE_2) is an important mediator of the pathogenesis of inflammatory diseases, achieved through activation of EP_2 receptors and subsequent protein kinase A (PKA)-dependent phosphorylation of GlyRα3.[4, 9, 13] It has also been reported that protein kinase C epsilon (PKCε) plays a pivotal role in hyperalgesic priming.[14] The PKA-mediated acute phase of hyperalgesia is evoked by PGE_2, while PKCε mediates the prolonged phase.[15–17] PGE_2 stimulates phosphorylated extracellular signal-regulated kinase (p-ERK) and interleukin (IL)-6 protein kinase pathways in DRG neurons.[18] However, a previous study found that phosphorylated pan-PKC, p38, and c-Jun N-terminal kinase (JNK) were not altered following long-acting PGE_2-analog treatment in DRG neurons.[18] The downstream

mechanism of GlyRs remain poorly understood. In our first experiment, we explored downstream proteins and signals mediated by GlyRs.

Adeno-associated virus (AAV) is a member of the parvovirus family that can be used to infect humans in clinical trials, and in experimental animal models.[19] The use of AAV vectors for gene therapy in human clinical trials has shown promise, as AAV generally causes a very mild immune response and long-term gene transfer, and there have been no reports of disease. For tissue tropism, the existence of a variety of serotypes makes AAV gene therapy more attractive, since they differ in infectivity rates and tissue specificity. Previous studies demonstrated extensive and effective transduction of the brain and spinal cord after AAV8 injection.[20, 21] The F11 neuron cell line possesses many properties seen in nociceptive DRG neuronal cells. The transient transfection efficiency is about 50% for F11 neuron cells.[22, 23] Compared with other cell lines, such as HEK293, F11 neuron cells are excellent proxies for the responses of real neuron cells. Therefore, we used AAV8 for GlyRα1/3 gene transfer in F11 neuron cells, and investigated the effects and roles of pAAV-GlyRα1/3 on cell cytotoxicity and the PGE_2-mediated inflammatory response.

Materials & Methods

Cell culture & bacteria

F11 cells was purchased from European Collection of Authenticated Cell Cultures (ECACC, 08062601) and was cultured in DMEM supplemented with 10% FBS (both Invitrogen) in a humidified atmosphere at 37 ℃ with 5% CO2. Escherichia coli DH5α strain was cultured at 37°C in Luria-Bertani (LB) broth medium supplied with kanamycin (50 ug/ml) and was used as a host in transformation. The institutional review board of Kaohsiung Medical University, Kaohsiung, Taiwan approved gene recombinant experiment in this study (KMU-106076).

pAAV-GlyRα1, pAAV-GlyRα3 recombinant vector construction

Firstly, total RNA was extracted from brain of SD rat, then was reversed to cDNA by using MMLV Reverse Transcription kit (Protech). GlyRα1, GlyRα3 fragments were PCR-amplified from rat cDNA using primers as following. Primers for GlyRα1 (forward: 5’-ACAGCGGCCGCACCATGTACAGCTTCAACACTCTG-3’, reverse: 5’-GGCGATATCTCACTTGTTGTGGACGTC-3’); primers for GlyRα3 (forward: 5’-ACAGCGGCCGCACCATGCCTTGGATAAGACTG-3’, reverse: 5’-GGCGATATCTTAATCTTGCTGATGATGAATG-3’). These two GlyRα fragments of PCR products were eluted and purified from Low Melting Point Agarose gels (ThermoFisher Scientific), then were ligated into pCR™-Blunt II-TOPO® vector by Zero Blunt® TOPO® PCR Cloning Kits (Invitrogen) to construct recombinant vectors pBlunt-GlyRα1, pBlunt-GlyRα3. Later, these two recombinant vectors were transfer to Escherichia coli DH5α competent cells by transformation. Extraction and purification of recombinant vectors pBlunt-GlyRα1, pBlunt-GlyRα3 from Escherichia coli DH5α cells was carried out with Presto™ Mini Plasmid Kit (PDH100, PDH300) (Geneaid, Taiwan). Accuracy of GlyRα1, GlyRα3 fragments which ligated with pCR™Blunt II-TOPO® vector was

further confirmed by PCR (primers for GlyRα1 forward: 5'-AAGAATTTCCCGATGGACGTA-3', reverse: 5'-GTAGTGCTTGGTGCAGTA-3'; primers for GlyRα3 (forward: 5'-AAACACTACAATACAGGAAAGTTTAC-3', reverse: 5'-CAGTGGTGATACCCAACG-3') and DNA sequencing. Secondary, Not I and EcoRV restriction enzymes were selected to cleaves pBlunt-GlyRα1, pBlunt-GlyRα3 recombinant DNA and pAAV-IRES-GFP expression vector. The restriction products were detected by Low Melting Point Agarose gels electrophoresed, eluted, purified and subjected to ligation reaction by T4 DNA ligase (BioLabs) to construct recombinant vectors pAAV-GlyRα1, pAAV-GlyRα3 (Fig. 1). Lastly, by transformation, these two recombinant vectors were then transfer to Escherichia coli DH5α competent cells. Recombinant vectors pAAV-GlyRα1, pAAV-GlyRα3 from Escherichia coli DH5α cells was extracted and purified again with Presto™ Mini Plasmid Kit (PDH100, PDH300) (Geneaid, Taiwan). Accuracy of GlyRα1, GlyRα3 fragments from pAAV-GlyRα1, pAAV-GlyRα3 were further confirmed by PCR (primers for GlyRα1 forward: 5'-AAGAATTTCCCGATGGACGTA-3', reverse: 5'-GTAGTGCTTGGTGCAGTA-3'; primers for GlyRα3 (forward: 5'-AAACACTACAATACAGGAAAGTTTAC-3', reverse: 5'-CAGTGGTGATACCCAACG-3') and DNA sequencing. The correct recombinant clones which containing pAAV-GlyRα1, or pAAV-GlyRα3 were store at -80℃ for further use.

pAAV-GlyRα1, pAAV-GlyRα3 transfection into F11 cells

Seeded F11 cells into a 6 well plate at a density of $3x10^5$ cells/well. When cell confluence reached 70%, transfected with pAAV, pAAV-GlyRα1 or pAAV-GlyRα3 (2 ug or 5 ug) recombinant vectors with a Lipofectamine® 2000 Transfection Reagent (Invitrogen) at 37˚C with 5% CO2 for 24, 48, 72 hours, respectively. Transfection efficiency was assayed by counting green fluorescent protein (GFP) emitted from pAAV-GlyRα1, or pAAV-GlyRα3 recombinant vectors inside the F11 cells.

Cell Viability assay

The effect of pAAV, pAAV-GlyRα1 or pAAV-GlyRα3 as well as PGE2 on F11 cells was determined by MTT assay. F11 cells ($7 x10^4$ cells/well) were seeded in the 24 well plate, after 24 hours culture, 2 ug pAAV, pAAV-GlyRα1, pAAV-GlyRα3 or lipofectamine only was used to transfect F11 cells for 48 hours. In addition, F11 cells treated with Prostaglandin E2 (PGE2, 100 uM) for 60 mins was included in the experiment. In the end, cell viability was assessed by the MTT assay kit (abcam) according to the manufacturer's protocol. Briefly, remove culture medium and MTT reagent was added into each well, incubated for 2~6 hours at 37ºC, remove MTT reagent then DMSO was added and incubated for 5 minutes. Supernatant was collected and absorbance was measured at OD 550~600 nm. F11 cells without transfection and lipofectamine treatment was used as control.

Western blots

To survey the effect of PGE2, or recombinant pAAV-GlyRα1 or pAAV-GlyRα3 on inducing pERK phosphorylation and activating transcription factor 3 (ATF-3) activation, 2 ug pAAV, or pAAV-GlyRα1 or pAAV-GlyRα3 was selected to transfect F11 cells ($3 x10^5$ cells/well), which seeded into the 6 well plate, for 48 hours. Serum free medium was replaced for another 24 hours, then F11 cells was treated with

PGE2 (100 uM) for 5, 15, 30 and 60 mins. F11 cells treated PGE2 alone or F11 cells transfected with pAAV, or pAAV-GlyRα1 or pAAV-GlyRα3 alone was also included in the present study. To investigate the pathway of PGE2 induced pERK phosphorylation, a glycine receptor antagonist Strychnine, EP2 receptor antagonist PF-04418948 was added for 24 hours. One hours after PGE2 administration cell pellets were harvested and pERK phosphorylation was measured. In western blotting, seeded cells as described above were harvested and were homogenized in RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Roche, Germany). The protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). 20 ug of total protein was loaded into 8% (w/v) sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The filters were incubated with rabbit monoclonal anti-Phospho-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, 4370p), rabbit monoclonal anti-p44/42 MAPK (Erk1/2) (Cell Signaling Technology, 4695p), rabbit monoclonal anti-phospho-p38 MAPK (mitogen-activated protein kinase; Cell Signaling Technology, Boston, MA, USA), rabbit monoclonal anti-p38 MAPK (Cell Signaling Technology), rabbit anti-ATF3 (NOVUS, NBP1-85816, USA), or mouse monoclonal anti-actin (MAB1501; Indianapolis, IN, USA) primary antibodies. This was followed by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). The intensity of each band was visualized with ECL Western blotting detection reagents (Amersham Biosciences, Tokyo, Japan). Protein expression was normalized using β-actin as an internal control and against the protein levels of the control group.

Measurement of cytokines by ELISA

To investigate the possible role of glycine receptors on modulating lipopolysaccharides (LPS) or complete Freund's adjuvant (CFA) induced cell inflammatory reaction. According to the transfection efficiency assay described above, 2 ug pAAV, pAAV-GlyRα1 or pAAV-GlyRα3 was selected to transfect F11 cells (3 x10^5 cells/well), which seeded into the 6 well plate, for 48 hours. Serum free medium was replaced for another 24 hours, then was treated with LPS (100 ng), or CFA (100 ng) for another 6 hours. Supernatant was collected for analyses of cytokines, including IL-1ß, tumor necrosis factor (TNF)-α, and IL-6 by ELISA (R&D Inc., Minneapolis, MN, USA). F11 cells without pAAV, pAAV-GlyRα1, or pAAV-GlyRα3 transfection or LPS/CFA treatment was used as control.

Statistical analysis

Results are presented as mean ± SE. Analytical statistics were performed using the SPSS (version 20) software package. Statistical significance was calculated by nonparametric Mann-Whitney U test and for pair-wise comparisons only. In some cases, ANOVA followed by Scheffe multiple post hoc test were used. Differences were considered statistically significant at *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

Results

pAAV/pAAV-GlyRα1/3 transfection does not induce cytotoxicity

The time schedule for measuring the transfection efficiency of pAAV-GlyR α1/pAAV-GlyR α3 is shown in Fig. 2A. Increased transfection efficiency was found after long-duration pAAV-GlyRα1 (Fig. 2B) or pAAV-GlyRα3 transfection (Fig. 2C). Therefore, transfection with 2 ug pAAV, pAAV-GlyRα1, or pAAV-GlyRα3 for 48 hours was used in our study, including for the MTT assay. Figure 3A shows the time schedule for measuring cell viability in the MTT assay. The viability of F11 cells transfected using pAAV, pAAV-GlyRα1, pAAV-GlyRα3, or lipofectamine alone, as well as those treated with PGE_2, was evaluated. Compared with the control, cell viability was significantly decreased in cells transfected with pAAV, pAAV-GlyRα1, or pAAV-GlyRα3, as well as those treated only with lipofectamine. However, cell viability was lower (but not significantly) in the pAAV and pAAV-GlyRα1/3 groups compared with the lipofectamine-treated group (Fig. 3B). This indicates that decreased cell viability in pAAV- or pAAV-GlyRα1/3-transfected groups was caused by the lipofectamine transfection reagent. However, cytotoxicity caused by pAAV/pAAV-GlyRα1/3 transfection cannot be ruled out completely. Furthermore, cell toxicity caused by PGE_2 treatment was not found in this study (Fig. 3B).

pAAV/pAAV-GlyRα1/3 transfection does not induce ERK1/2 phosphorylation and ATF-3 activation

Paav-glyrα3 Suppresses Pge-induced Erk1/2 Phosphorylation

The time schedule of PGE_2 administration and cell collection for measuring pERK phosphorylation and ATF-3 activation is shown in Fig. 4A. The western blotting results indicated that phosphorylation of ERK1/2, but not phosphorylated p38 (p-p38; data not shown), increased significantly 30–60 minutes after administration of PGE_2 (100 ng) (Figs. 4B, C). Importantly, PGE_2 did not correlate with neuron cell injury or ATF-3 activation (Figs. 4B, D).

The time schedule of 48-hour pAAV or pAAV-GlyRα1/3 transfection, and cell collection for measuring ERK phosphorylation and ATF-3 activation, is shown in Figure. 5A. We found that lipofectamine induced significant ERK phosphorylation (Figs. 5B, C) and ATF-3 activation (Figs. 5B, D), although pAAV-GlyRα3 transfection was superior for inducing ERK phosphorylation and ATF-3 activation compared with the normal control (Figs. 5B–D). However, there was no significant difference between the lipofectamine-treated group and pAAV-GlyRα3 transfection groups. This indicates that neither pAAV nor pAAV-GlyRα1/3 transfection causes ERK phosphorylation or ATF-3 activation. We also examined the effect of vectors, including pAAV-GlyRα1 and pAAV-GlyRα3, on PGE_2-induced ERK1/2 phosphorylation (Fig. 6A). We found that PGE_2 administration induced ERK1/2 phosphorylation; however, pre-transfected pAAV-GlyRα3 (but not pAAV-GlyRα1) significantly suppressed PGE_2-induced ERK1/2 phosphorylation (Figs. 6B, C).

Blocking The Glycine Receptor Inhibits Pge-induced Perk1/2 Phosphorylation

Strychnine is an inhibitor of postsynaptic GlyRs. We examined whether the EP_2 or glycine receptor was responsible for PGE_2-induced ERK1/2 phosphorylation in F11 cells. Figure 7A shows the time schedule for application of the EP_2- and glycine-receptor antagonists, PF-04418948 and strychnine, respectively. The results showed that PF-04418948 and strychnine downregulate PGE_2-induced ERK1/2 phosphorylation, suggesting that GlyRs (except EP_2) are essential to PGE_2-induced ERK1/2 phosphorylation. In addition, the broad-spectrum PKC inhibitor G06983 given 30 minutes before PGE_2 treatment also led to a significant reduction in PGE_2-induced ERK1/2 phosphorylation (Figs. 7B, C).

LPS and CFA do not induce cytokine expression in F11 cells

We investigated the effects of pAAV, pAAV-GlyRα1, and pAAV-GlyRα3 on LPS

and CFA induced cytokine expression in F11 cells. The time schedule for the ELISA experiment is shown in Fig. 8A. We found that TNF-α (Fig. 8B), IL-1ß (Fig. 8C), and Il-6 (Fig. 8D) were not induced in LPS/CFA-treated F11 cells, or those transfected with pAAV, pAAV-GlyRα1, or pAAV-GlyRα3.

Discussion

We found that pAAV/pAAV-GlyRα1/3 transfection did not induce significant cell cytotoxicity, ERK1/2 phosphorylation, or ATF-3 activation. In addition, PGE_2-induced ERK1/2 phosphorylation was repressed in F11 cells by the expression of pAAV-GlyRα3.

The transfection reagent Lipofectamine 2000 can induce cell damage.[24] We used lipofectamine in our transfection experiments and found significantly decreased cell viability following pAAV or pAAV-GlyRα1/3 transfection, caused by the reagent. This indicates that pAAV/pAAV-GlyRα1/3 is safe for neuron cell transfection in vitro and should be suitable for use in animal transfection.

PGE_2 has a short lifespan (usually less than 2.5 hours).[25] Similar to previous studies, exogenous PGE_2 was shown to directly induce ERK1/2 (but not p38) phosphorylation in DRG neurons[18] and other non-neuron cell types[18],[26] Zhao et al.[27] found a PGE_2-dependent, ERK1/2-regulated microglia-neuron signaling pathway that mediated the microglial component of pain maintenance.

We found that the ERK phosphorylation in pAAV-GlyRα3 transfection was caused by lipofectamine. Furthermore, our in vitro results indicated that exogenous pAAV-GlyRα3 administration can suppress PGE_2-induced ERK1/2 phosphorylation in F11 neuron cells. Other studies have shown that specific GlyR subtypes play a key role in different diseases, for example, the GlyRα1 subtype is associated with tumorigenesis and alcoholism,[28, 29] and the GlyRα3 subtype is associated with inflammatory hyperalgesia.[1, 9, 12]

PKC-dependent phosphorylation of p38 and ERK have been reported.[30] The phosphorylation of ERK occurs in a PKA-independent manner.[31] Conversely, Chen et al. showed that PKA stimulated p38 and ERK phosphorylation in breast adipose fibroblasts.[26] Furthermore, a previous study found that pERK1/2 increased significantly in DRG neurons 8 hours after PGE_2 exposure; co-treatments of PGE_2 with inhibitors of pan-PKA, pan-PKC, and ERK/mitogen-activated protein kinase (MAPK) significantly suppressed PGE_2-induced IL-6 expression.[18]

Our study showed that pAAV-GlyRα3 transfection or administration of glycine-receptor antagonist (strychnine) can downregulate PGE_2-induced ERK1/2 phosphorylation in F11 neuron cells. There are two possible explanations for this. First, ERK1/2 phosphorylation is also controlled by GlyRs through the PKA/PKC-dependent pathway.[9, 26, 32] Second, the glycine-receptor structure changes during strychnine binding, as does the internal domain binding site of the PGE_2-dependent PKA/PKC pathway.[33, 34] A previous study showed that GlyRa3 architecture changed after strychnine binding. For an agonist or antagonist to bind with and affect the state of the channel, the signal must be transduced across the extracellular domain and transmembrane domain interface.[34] Further studies are needed to elucidate the GlyR signaling pathways and identify additional potential molecular targets for inflammatory pain inhibition.

The pro-inflammatory cytokines TNF-α[35] and IL-6[18, 36] may be upregulated in DRG neurons after peripheral nerve injury. A previous study used a partial sciatic-nerve ligated model to test IL-6 in DRG neurons, and found upregulation of IL-6 in DRG neurons following nerve injury.[18] Furthermore, increased IL-6 expression shifted from small- to medium- and large-sized damaged DRG neurons. Nerve injury models have also induced neuronal cell death, thereby inducing more pro-inflammation cytokines, such as IL-6.[18, 27] Both LPS[37–39] and CFA[40, 41] are strong inflammatory mediators and can induce PGE_2 synthesis in animal models. Our results showed that the expression of TNF-α, IL-1ß, and IL-6 did not change in F11 cells treated by LPS/CFA or transfected with pAAV, pAAV-GlyRα1, or pAAV-GlyRα3. Hashemian et al. also used F11 neuron cells and found that LPS induced modest increases in IL-6 and COX2 expression, but did not induce significant TNF-α expression.[42]

A previous ex vivo study treated cultured sensory ganglion explants with a stabilized, long-acting PGE_2 analog ($dmPGE_2$) and showed that after high-dose $dmPGE_2$ (100 μM) treatment, IL-6 expression increased significantly at 20 and 24 hours[18]. However, sensory ganglion explants were used, which differ from the F11 neuron cell line chosen for the present study. Except for neuron cells, sensory ganglion explants include satellite glial cells, which are surrounded and can modulate neuron cell function. Satellite glial cells are also important immune regulators and can produce inflammatory mediators, such as prostaglandins, IL-6, and TNF-α. In addition, in the nervous system, it has been suggested that cytokines are secreted by peripheral immune cells, microglia, astrocytes, and neurons.

Conclusions

The present study is the first to use AAV as a glycine receptor (pAAV-GlyRs) vector to infect neuron cells. Though PGE_2-induced ERK1/2 phosphorylation in F11 neuron cells. Antagonists of prostaglandin EP_2 receptor, PKC, and glycine receptor can inhibit PGE_2-induced ERK1/2 phosphorylation. This study found pAAV/pAAV-GlyRα1/3 transfection does not induce cell cytotoxicity, ERK1/2 phosphorylation, or ATF-3 activation in neuron cells. Furthermore, pre-transfected pAAV-GlyRα3 can significantly suppress PGE_2-induced ERK1/2 phosphorylation. We suggest that PGE_2-induced ERK1/2 phosphorylation can be modulated by GlyRα3. pAAV/pAAV-GlyRα1/3 recombinant vectors could be a good model for studying glycine-receptor function in neuron cells and are probably suitable for animal infection models.

Abbreviations

GlyRs, Glycine receptors; DRG, dorsal root ganglion; PGE2, Prostaglandin E2; PKA, protein kinase A; PKCε, protein kinase C epsilon; p-ERK, phosphorylated extracellular signal-regulated kinase; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; AAV, Adeno-associated virus; GFP, green fluorescent protein; ATF-3, activating transcription factor 3; LPS, lipopolysaccharides; CFA, complete Freund's adjuvant; TNF-α,tumor necrosis factor α; MAPK, mitogen-activated protein kinase.

Declarations

Ethics approval and consent to participate: The institutional review board of Kaohsiung Medical University, Kaohsiung, Taiwan approved gene recombinant experiment in this study (KMU-106076).

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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References

1. Lynch JW. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev. 2004;84:1051–95.
2. Betz H, Langosch D, Hoch W, Prior P, Pribilla I, Kuhse J, Schmieden V, Malosio ML, Matzenbach B, Holzinger F, et al. Structure and expression of inhibitory glycine receptors. Adv Exp Med Biol. 1991;287:421–9.
3. Dutertre S, Becker CM, Betz H. Inhibitory glycine receptors: an update. J Biol Chem. 2012;287:40216–23.
4. Moraga-Cid G, San Martin VP, Lara CO, Munoz B, Marileo AM, Sazo A, Munoz-Montesino C, Fuentealba J, Castro PA, Guzman L, et al. Modulation of glycine receptor single-channel conductance by intracellular phosphorylation. Sci Rep. 2020;10:4804.
5. Bode A, Lynch JW. The impact of human hyperekplexia mutations on glycine receptor structure and function. Mol Brain. 2014;7:2.
6. Winkelmann A, Maggio N, Eller J, Caliskan G, Semtner M, Haussler U, Juttner R, Dugladze T, Smolinsky B, Kowalczyk S, et al. Changes in neural network homeostasis trigger neuropsychiatric symptoms. J Clin Invest. 2014;124:696–711.
7. Pilorge M, Fassier C, Le Corronc H, Potey A, Bai J, De Gois S, Delaby E, Assouline B, Guinchat V, Devillard F, et al. Genetic and functional analyses demonstrate a role for abnormal glycinergic signaling in autism. Mol Psychiatry. 2016;21:936–45.
8. Zeilhofer HU. The glycinergic control of spinal pain processing. Cell Mol Life Sci. 2005;62:2027–35.
9. Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, et al. GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science. 2004;304:884–7.
10. Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU. PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci. 2002;5:34–40.
11. Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Muller U, Zeilhofer HU. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest. 2005;115:673–9.
12. Hosl K, Reinold H, Harvey RJ, Muller U, Narumiya S, Zeilhofer HU. Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor alpha3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection. Pain. 2006;126:46–53.
13. Isensee J, Diskar M, Waldherr S, Buschow R, Hasenauer J, Prinz A, Allgower F, Herberg FW, Hucho T. Pain modulators regulate the dynamics of PKA-RII phosphorylation in subgroups of sensory neurons. J Cell Sci. 2014;127:216–29.
14. Reichling DB, Levine JD. Critical role of nociceptor plasticity in chronic pain. Trends Neurosci. 2009;32:611–8.
15. Dina OA, Khasar SG, Gear RW, Levine JD. Activation of Gi induces mechanical hyperalgesia poststress or inflammation. Neuroscience. 2009;160:501–7.

16. Khasar SG, Burkham J, Dina OA, Brown AS, Bogen O, Alessandri-Haber N, Green PG, Reichling DB, Levine JD. Stress induces a switch of intracellular signaling in sensory neurons in a model of generalized pain. J Neurosci. 2008;28:5721–30.
17. Aley KO, Messing RO, Mochly-Rosen D, Levine JD. Chronic hypersensitivity for inflammatory nociceptor sensitization mediated by the epsilon isozyme of protein kinase C. J Neurosci. 2000;20:4680–5.
18. St-Jacques B, Ma W. Role of prostaglandin E2 in the synthesis of the pro-inflammatory cytokine interleukin-6 in primary sensory neurons: an in vivo and in vitro study. J Neurochem. 2011;118:841–54.
19. Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev. 2008;21:583–93.
20. Klein RL, Dayton RD, Tatom JB, Henderson KM, Henning PP. AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. Mol Ther. 2008;16:89–96.
21. Ayers JI, Fromholt S, Sinyavskaya O, Siemienski Z, Rosario AM, Li A, Crosby KW, Cruz PE, DiNunno NM, Janus C, et al. Widespread and efficient transduction of spinal cord and brain following neonatal AAV injection and potential disease modifying effect in ALS mice. Mol Ther. 2015;23:53–62.
22. Mahapatra NR, Mahata M, Ghosh S, Gayen JR, O'Connor DT, Mahata SK. Molecular basis of neuroendocrine cell type-specific expression of the chromogranin B gene: Crucial role of the transcription factors CREB, AP-2, Egr-1 and Sp1. J Neurochem. 2006;99:119–33.
23. Jahnel R, Dreger M, Gillen C, Bender O, Kurreck J, Hucho F. Biochemical characterization of the vanilloid receptor 1 expressed in a dorsal root ganglia derived cell line. Eur J Biochem. 2001;268:5489–96.
24. Zhong YQ, Wei J, Fu YR, Shao J, Liang YW, Lin YH, Liu J, Zhu ZH. [Toxicity of cationic liposome Lipofectamine 2000 in human pancreatic cancer Capan-2 cells]. Nan Fang Yi Ke Da Xue Xue Bao. 2008;28:1981–4.
25. St-Jacques B, Ma W. Peripheral prostaglandin E2 prolongs the sensitization of nociceptive dorsal root ganglion neurons possibly by facilitating the synthesis and anterograde axonal trafficking of EP4 receptors. Exp Neurol. 2014;261:354–66.
26. Chen D, Reierstad S, Lin Z, Lu M, Brooks C, Li N, Innes J, Bulun SE. Prostaglandin E(2) induces breast cancer related aromatase promoters via activation of p38 and c-Jun NH(2)-terminal kinase in adipose fibroblasts. Cancer Res. 2007;67:8914–22.
27. Zhao P, Waxman SG, Hains BC. Extracellular signal-regulated kinase-regulated microglia-neuron signaling by prostaglandin E2 contributes to pain after spinal cord injury. J Neurosci. 2007;27:2357–68.
28. Forstera B, Dzaye O, Winkelmann A, Semtner M, Benedetti B, Markovic DS, Synowitz M, Wend P, Fahling M, Junier MP, et al. Intracellular glycine receptor function facilitates glioma formation in vivo. J Cell Sci. 2014;127:3687–98.

29. Aguayo LG, Castro P, Mariqueo T, Munoz B, Xiong W, Zhang L, Lovinger DM, Homanics GE. Altered sedative effects of ethanol in mice with alpha1 glycine receptor subunits that are insensitive to Gbetagamma modulation. Neuropsychopharmacology. 2014;39:2538–48.
30. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev. 2012;92:689–737.
31. Laroche-Joubert N, Marsy S, Michelet S, Imbert-Teboul M, Doucet A. Protein kinase A-independent activation of ERK and H,K-ATPase by cAMP in native kidney cells: role of Epac I. J Biol Chem. 2002;277:18598–604.
32. Breitinger U, Bahnassawy LM, Janzen D, Roemer V, Becker CM, Villmann C, Breitinger HG. PKA and PKC Modulators Affect Ion Channel Function and Internalization of Recombinant Alpha1 and Alpha1-Beta Glycine Receptors. Front Mol Neurosci. 2018;11:154.
33. Han L, Talwar S, Wang Q, Shan Q, Lynch JW. Phosphorylation of alpha3 glycine receptors induces a conformational change in the glycine-binding site. ACS Chem Neurosci. 2013;4:1361–70.
34. Huang X, Chen H, Michelsen K, Schneider S, Shaffer PL. Crystal structure of human glycine receptor-alpha3 bound to antagonist strychnine. Nature. 2015;526:277–80.
35. Wei XH, Zang Y, Wu CY, Xu JT, Xin WJ, Liu XG. Peri-sciatic administration of recombinant rat TNF-alpha induces mechanical allodynia via upregulation of TNF-alpha in dorsal root ganglia and in spinal dorsal horn: the role of NF-kappa B pathway. Exp Neurol. 2007;205:471–84.
36. Lee KM, Jeon SM, Cho HJ. Tumor necrosis factor receptor 1 induces interleukin-6 upregulation through NF-kappaB in a rat neuropathic pain model. Eur J Pain. 2009;13:794–806.
37. Gray A, Maguire T, Schloss R, Yarmush ML. Identification of IL-1beta and LPS as optimal activators of monolayer and alginate-encapsulated mesenchymal stromal cell immunomodulation using design of experiments and statistical methods. Biotechnol Prog. 2015;31:1058–70.
38. Li Y, Ji A, Weihe E, Schafer MK. Cell-specific expression and lipopolysaccharide-induced regulation of tumor necrosis factor alpha (TNFalpha) and TNF receptors in rat dorsal root ganglion. J Neurosci. 2004;24:9623–31.
39. Tse KH, Chow KB, Leung WK, Wong YH, Wise H. Lipopolysaccharide differentially modulates expression of cytokines and cyclooxygenases in dorsal root ganglion cells via Toll-like receptor-4 dependent pathways. Neuroscience. 2014;267:241–51.
40. Fang JF, Liang Y, Du JY, Fang JQ. Transcutaneous electrical nerve stimulation attenuates CFA-induced hyperalgesia and inhibits spinal ERK1/2-COX-2 pathway activation in rats. BMC Complement Altern Med. 2013;13:134.
41. Basting RT, Spindola HM, Sousa IMO, Queiroz NCA, Trigo JR, de Carvalho JE, Foglio MA. Pterodon pubescens and Cordia verbenacea association promotes a synergistic response in antinociceptive model and improves the anti-inflammatory results in animal models. Biomed Pharmacother. 2019;112:108693.
42. Hashemian S, Alhouayek M, Fowler CJ. TLR4 receptor expression and function in F11 dorsal root ganglion x neuroblastoma hybrid cells. Innate Immun. 2017;23:687–96.

Figures

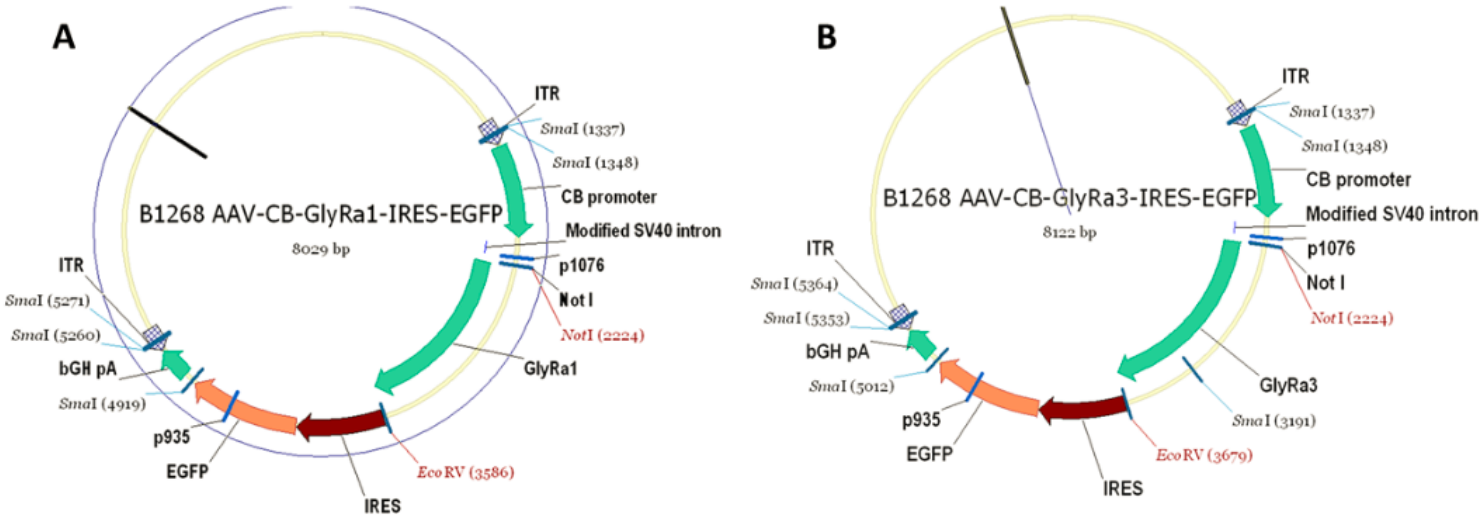
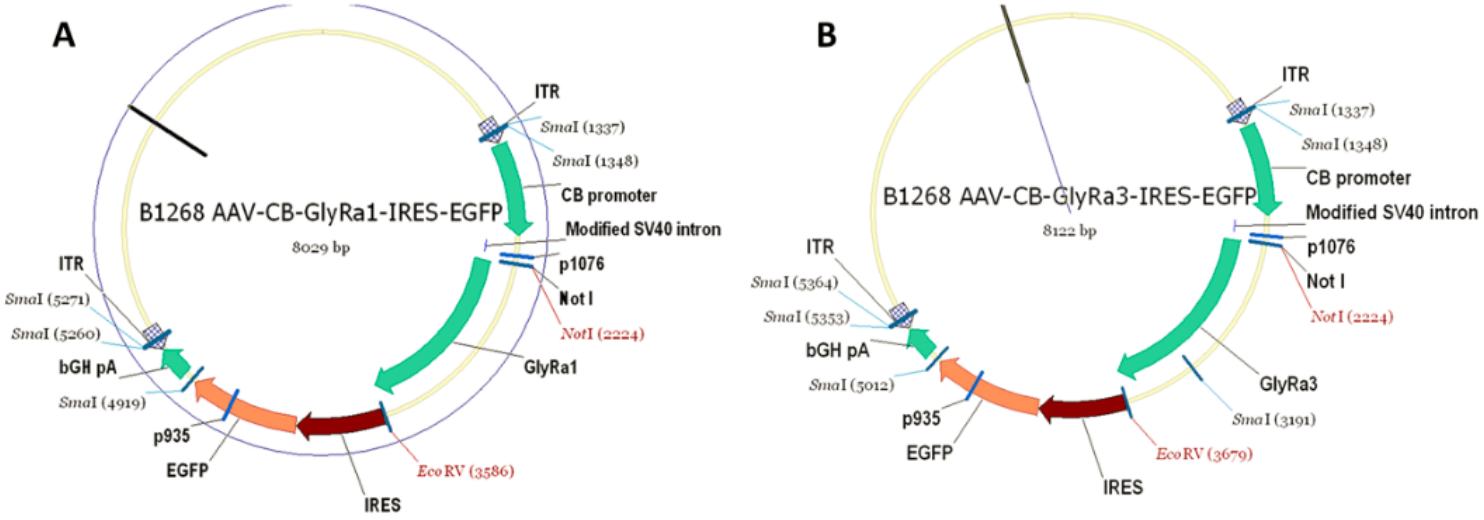


Figure 1

(A) pAAV-GlyRα1, (B) pAAV-GlyRα3 recombinant vector.



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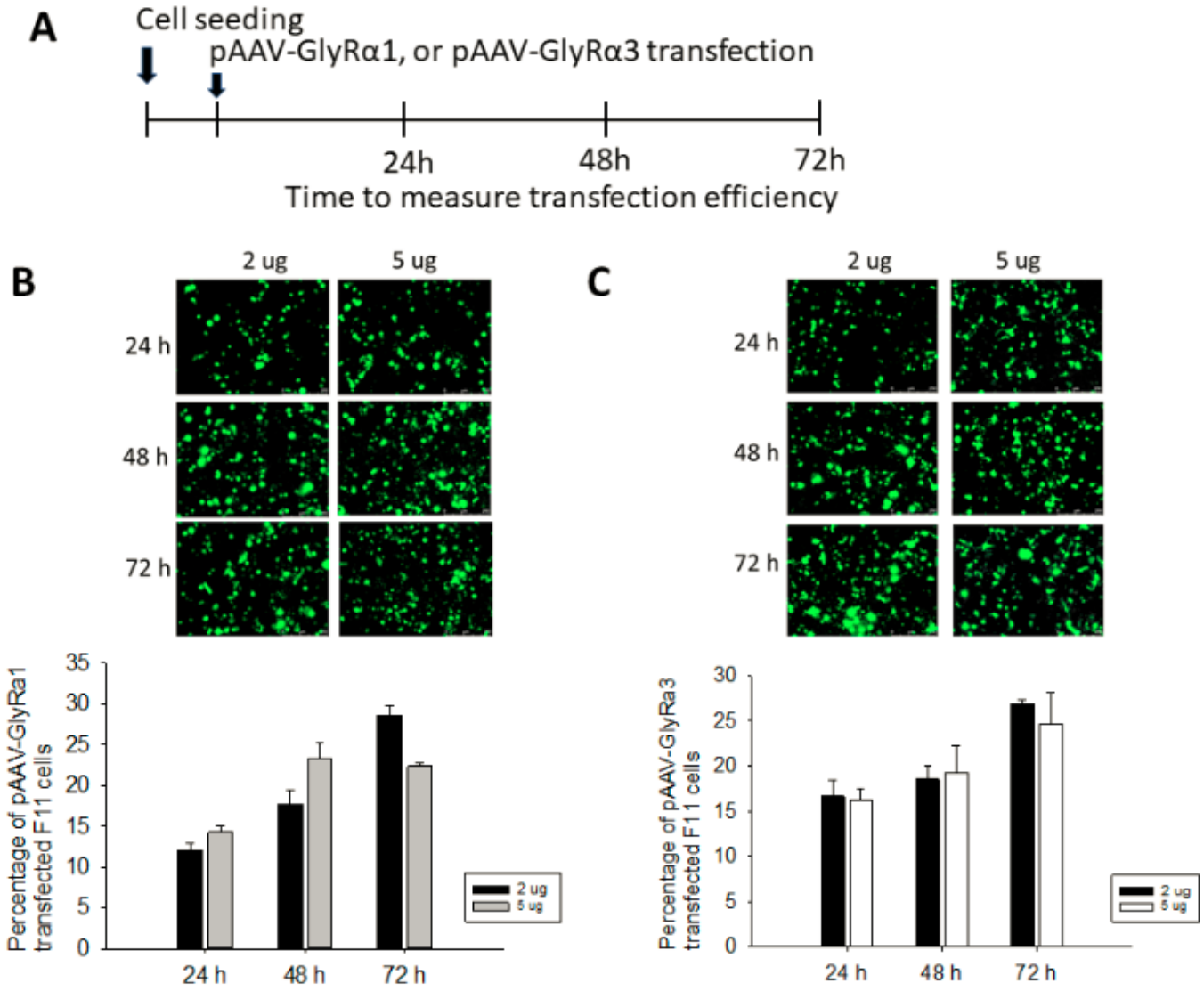
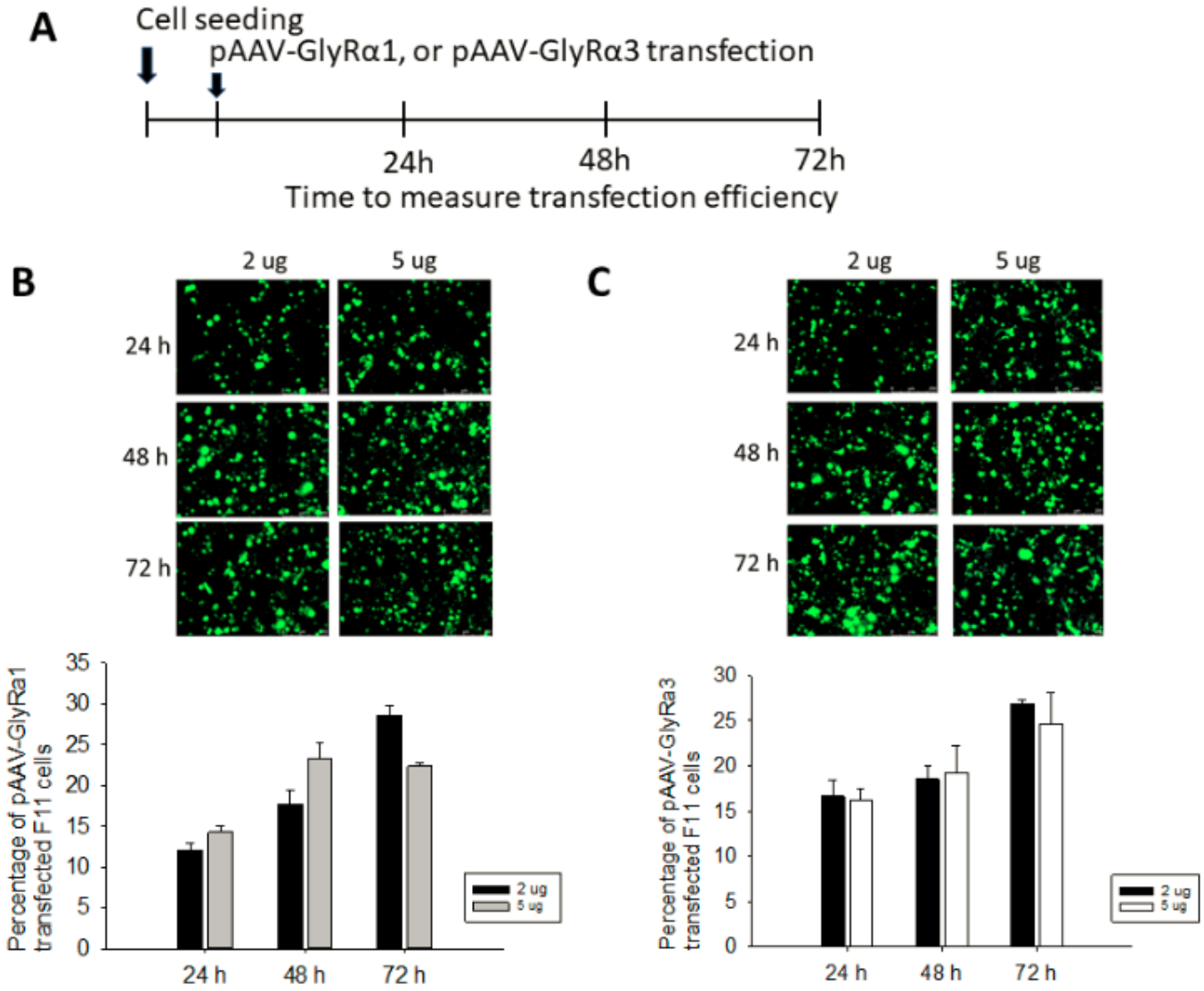


Figure 2

(A) Time schedule to measure transfection efficiency of pAAV-GlyR α1/pAAV-GlyR α3. F11 cells were transfected with 2 ug or 5 ug either (B) pAAV-GlyRα1 or (C) pAAV-GlyRα3 and the GFP green fluorescence were measured after transfection for 24, 48, 72 hours. Data are presented as the percentage from three independent experiments.



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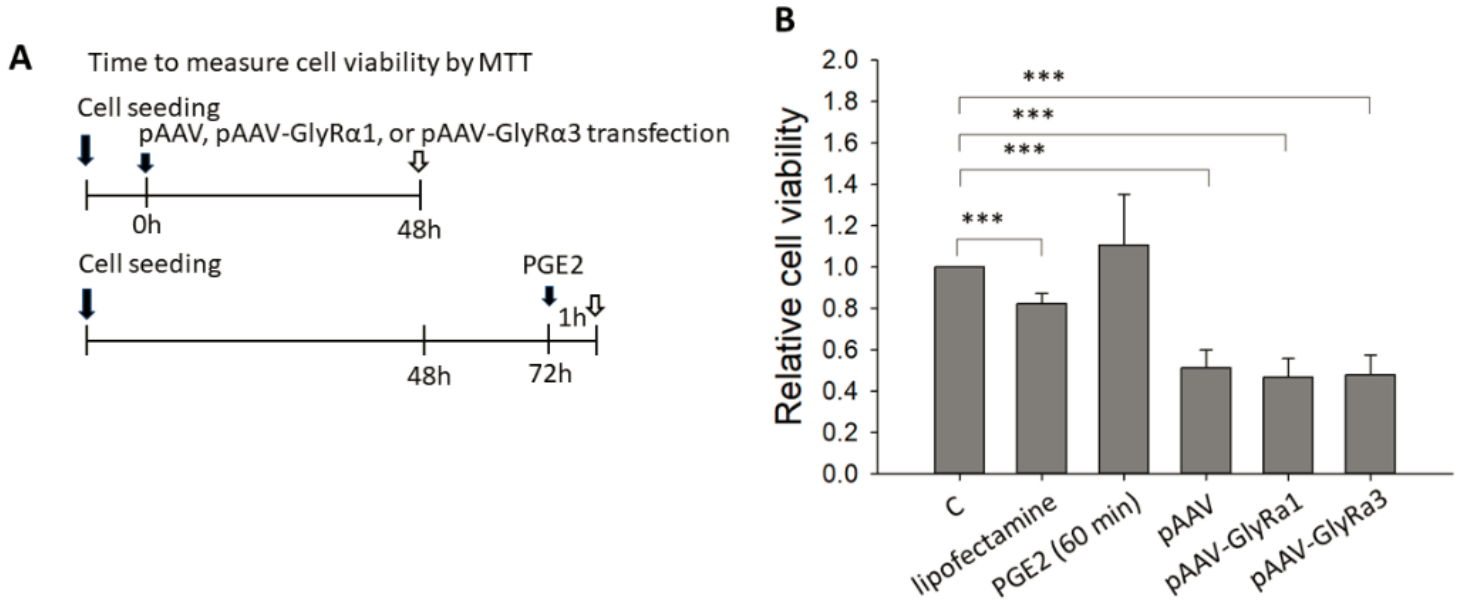
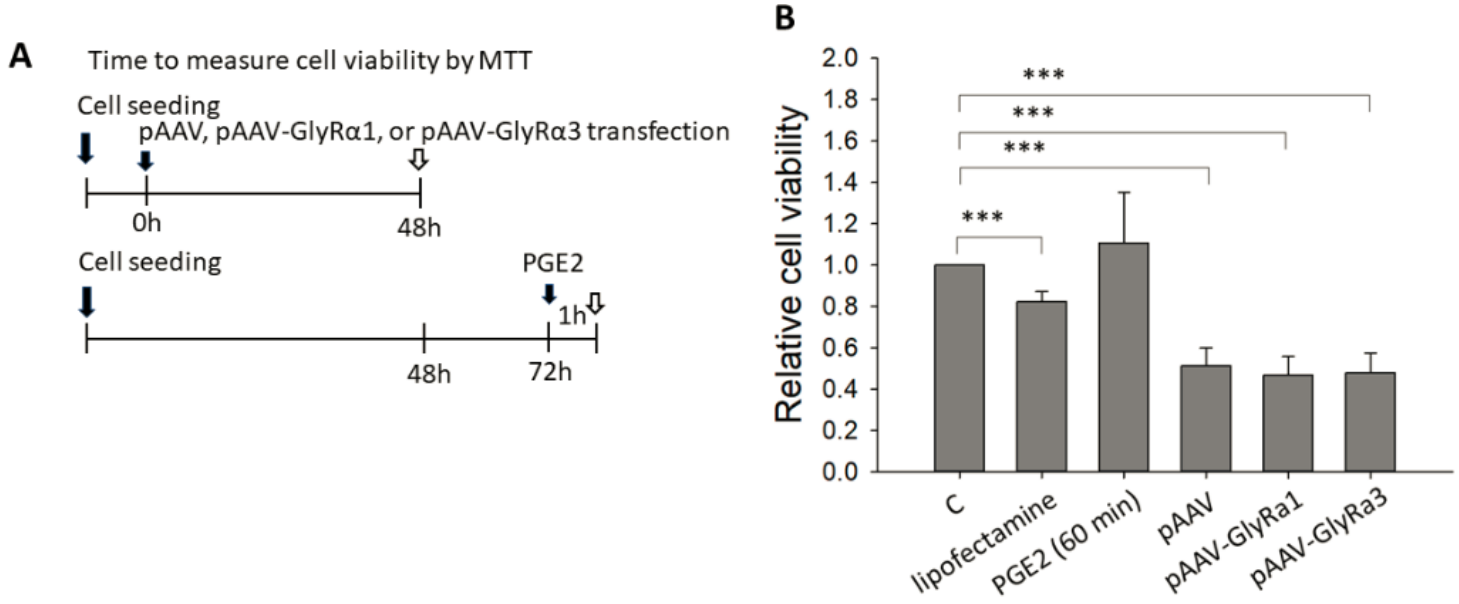


Figure 3

MTT assay was used to observe the cell viability in response to pAAV, pAAV-GlyRα1, pAAV-GlyRα3 transfection or PGE2 treatment. (A) Time schedule to measure cell viability by MTT assay. F11 cells was transfected with pAAV, pAAV-GlyRα1, or pAAV-GlyRα3 for 48 hours. In addition, culturing F11 cells for 48 hours, following replaced serum free medium for another 24 hours then treated with PGE2 for 60 mins. In the end, these cells were harvested for MTT assay. (B) Relative cell viability was shown. F11 cells grew in lipofetamine free culture medium was used as control. White arrow indicated the time cell collected to measure cell viability. Data are presented from two to three independent experiments. ***p< 0.001 vs. control.



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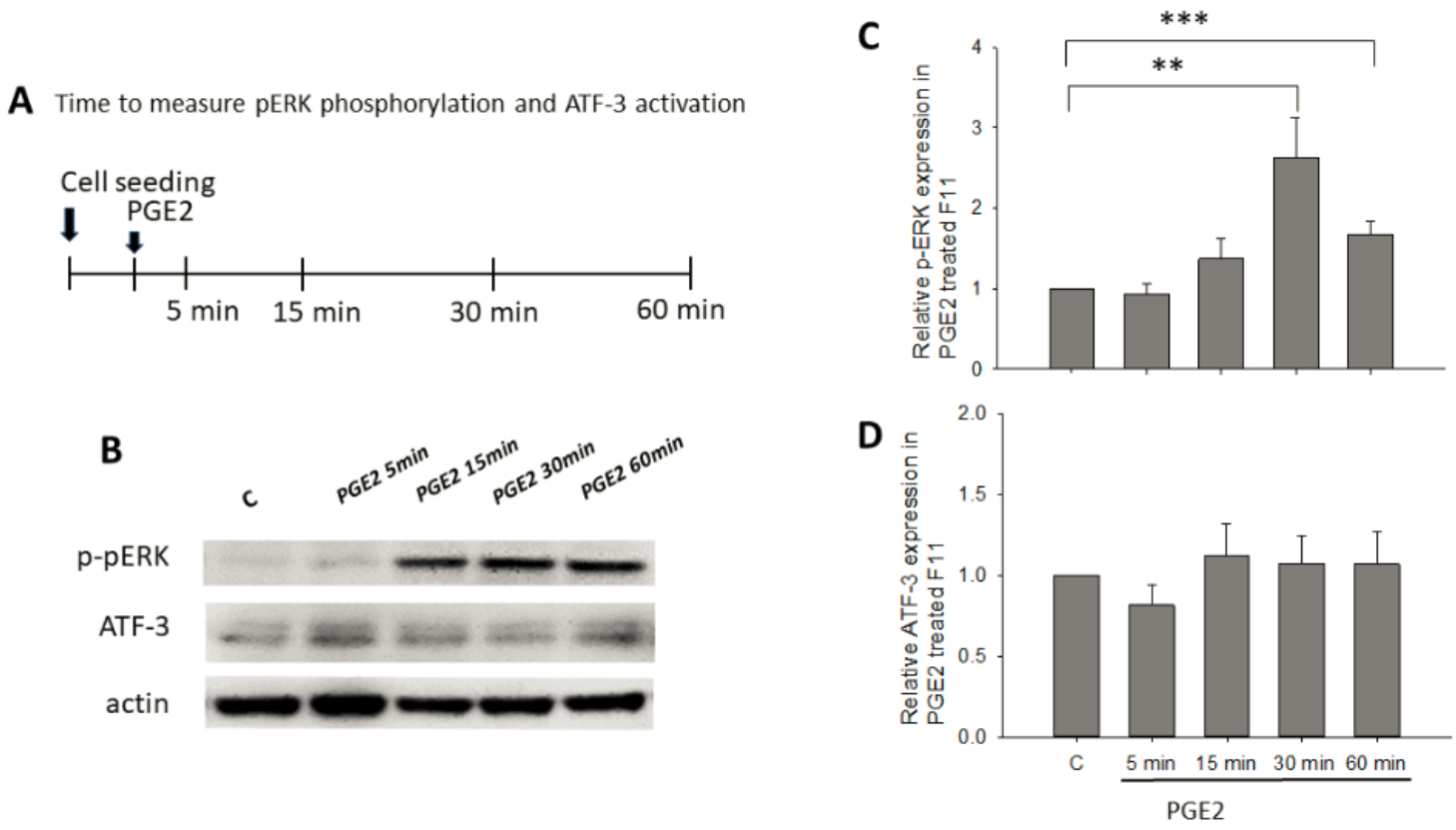
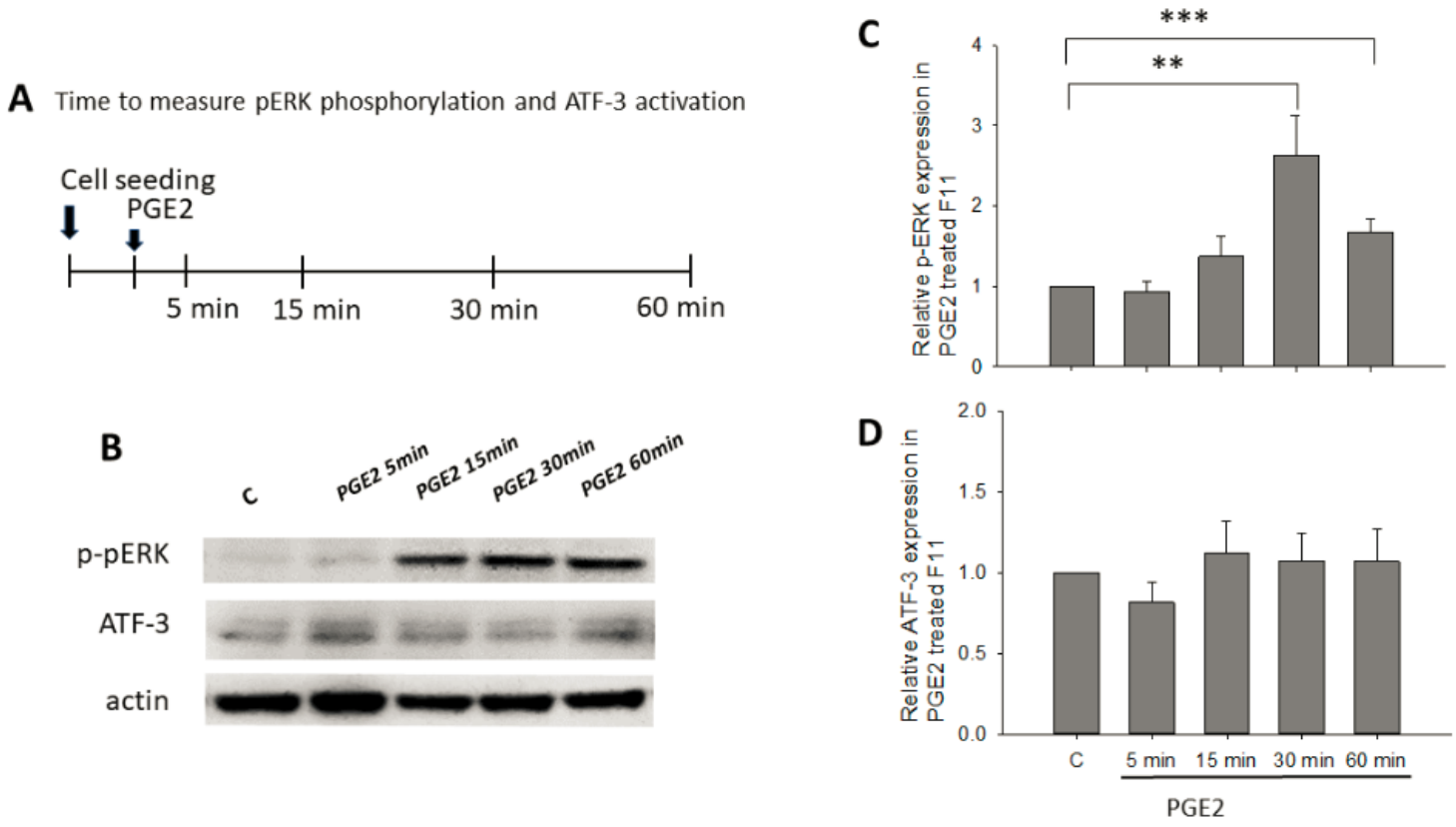


Figure 4

Effect of PGE2 (100 uM) on pERK phosphorylation and ATF-3 activation in F11 cells. (A) Time schedule of PGE2 administration and cell collection time to measure pERK phosphorylation and ATF-3 activation by western blotting. F11 cells seeded into the 6 well plate, 24 hours later, PGE2 was applied for 5, 15, 30 or 60 mins, respectively. Finally, these treated cells were harvested for protein extraction. (B) Western blotting images and quantitative evaluation of (C) p-pERK, (D) ATF-3 activation in PGE2 treated F11 cells were measured. Data are presented from five independent experiments. *p<0.05, **p< 0.01, and ***p< 0.001, One-way ANOVA.



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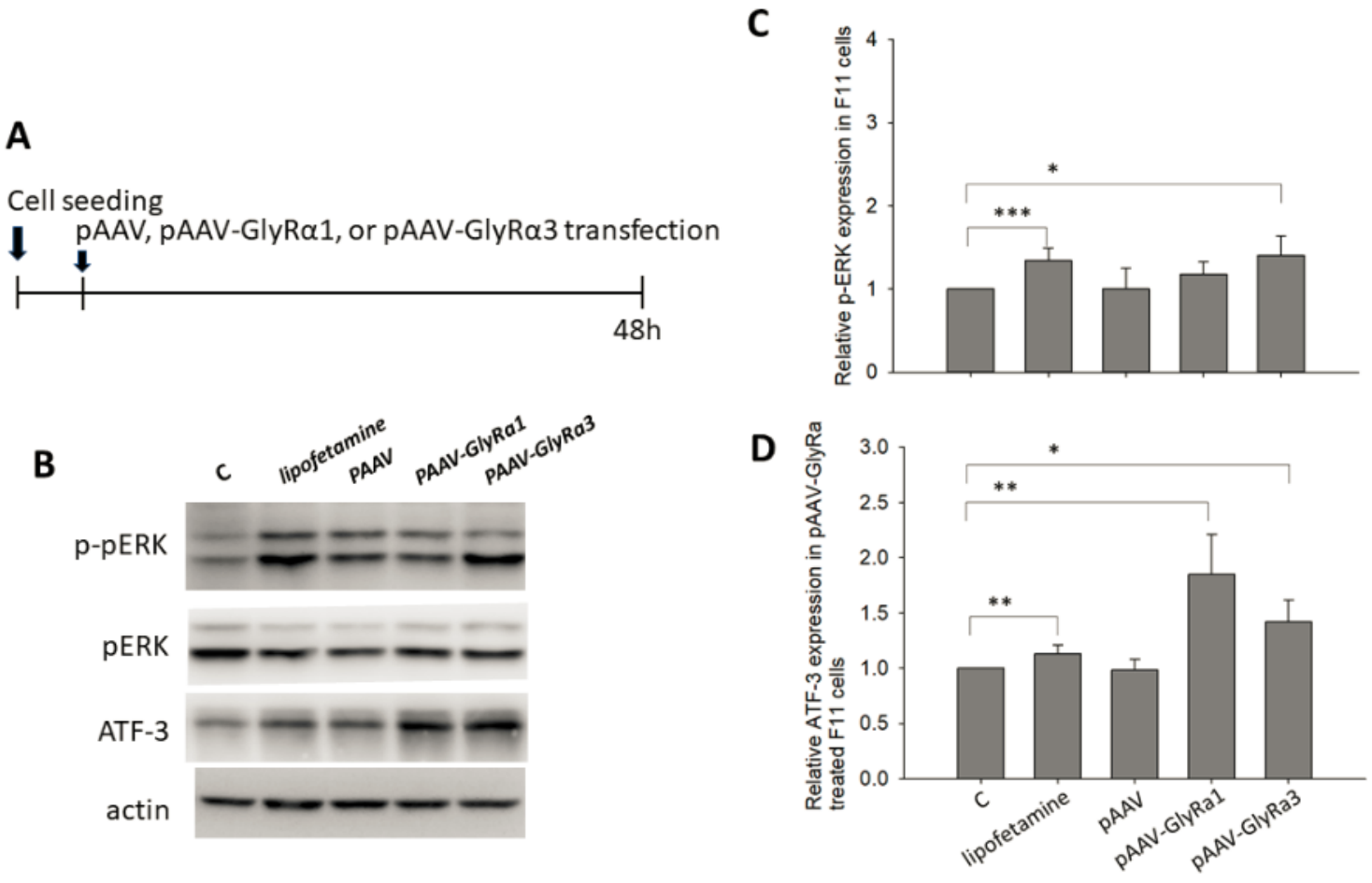

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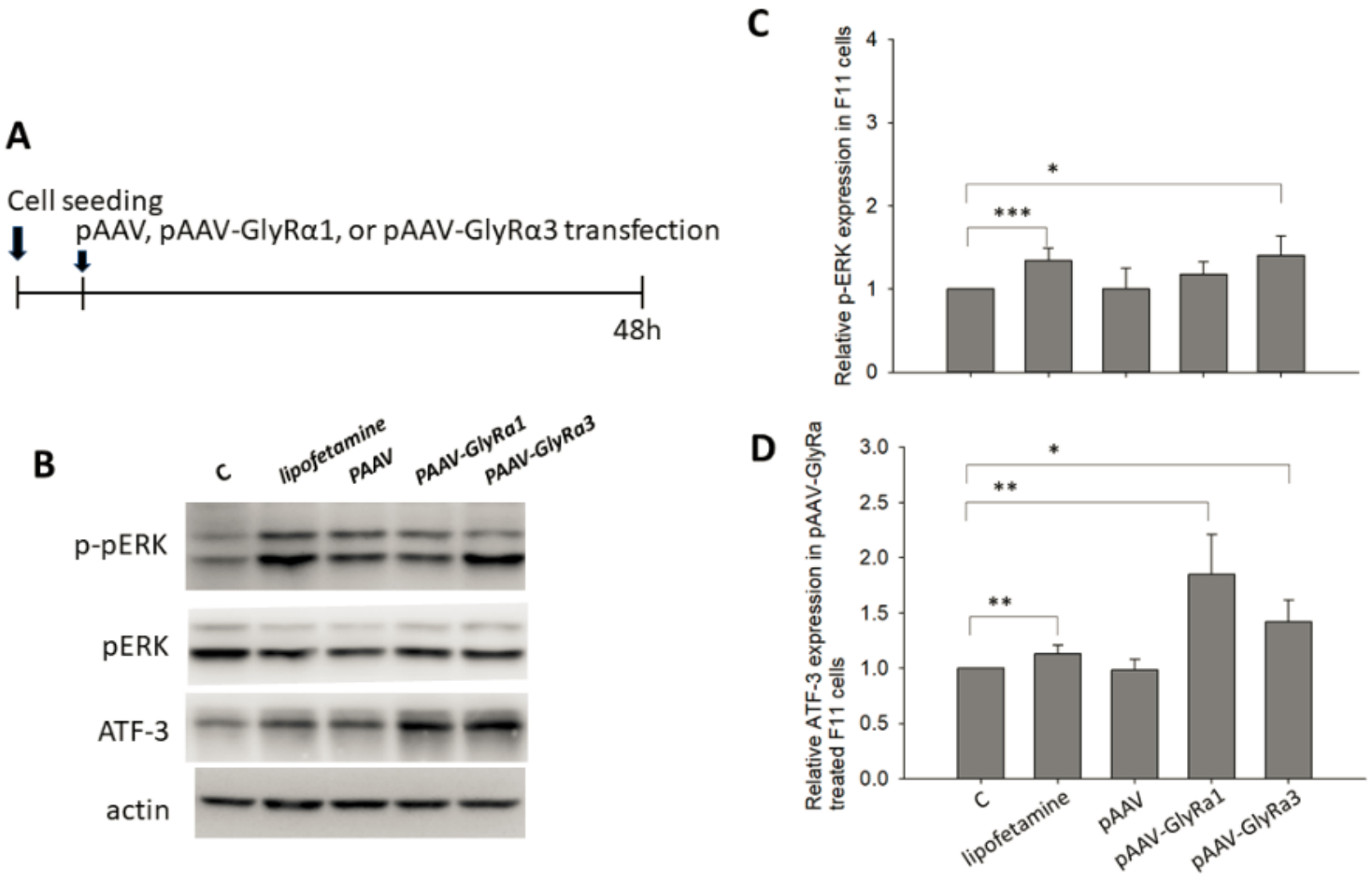

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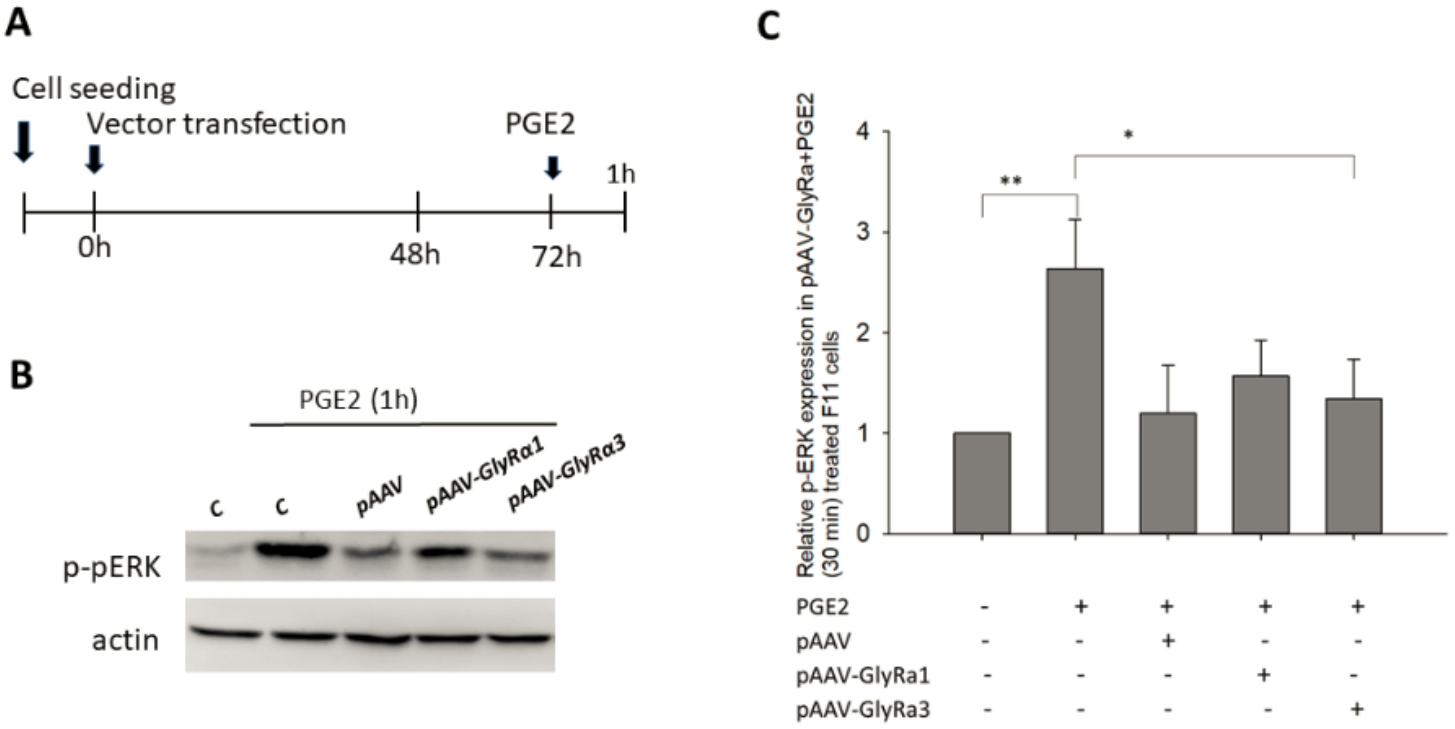
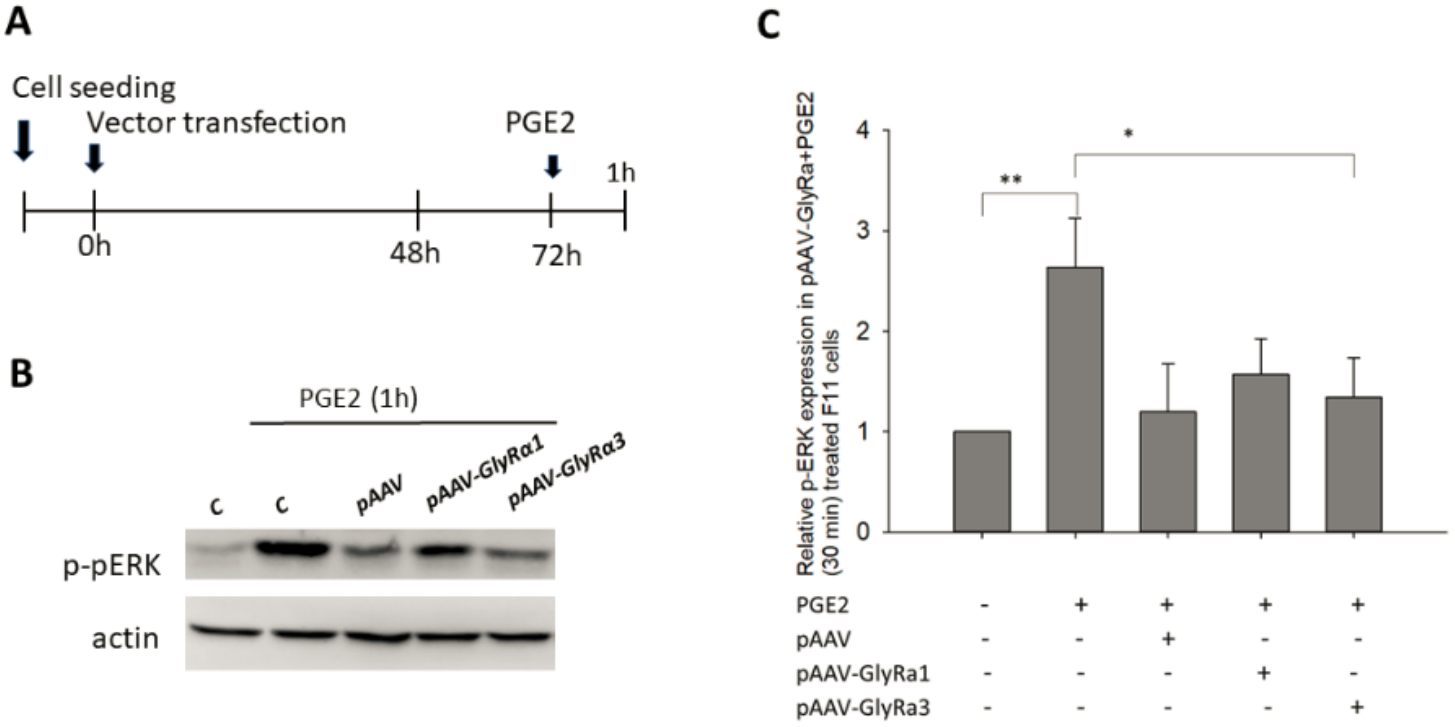


Figure 6

Effect of pAAV-GlyRα1/3 transfection on PGE2 (100 uM) induced ERK phosphorylation in F11 cells. (A) F11 cells were transfected with pAAV, pAAV-GlyR α1 or pAAV-GlyR α3 for 48 hours. Replaced serum free medium for another 24 hours then treated with PGE2 for 60 minutes. Cells were harvested for (B) western blotting of pERK phosphorylation, (C) quantification of pERK phosphorylation in F11 cells was shown. F11 cells without vector transfection or PGE2 treatment was used as control. All data were expressed as fold change. *p<0.05, **p<0.01.



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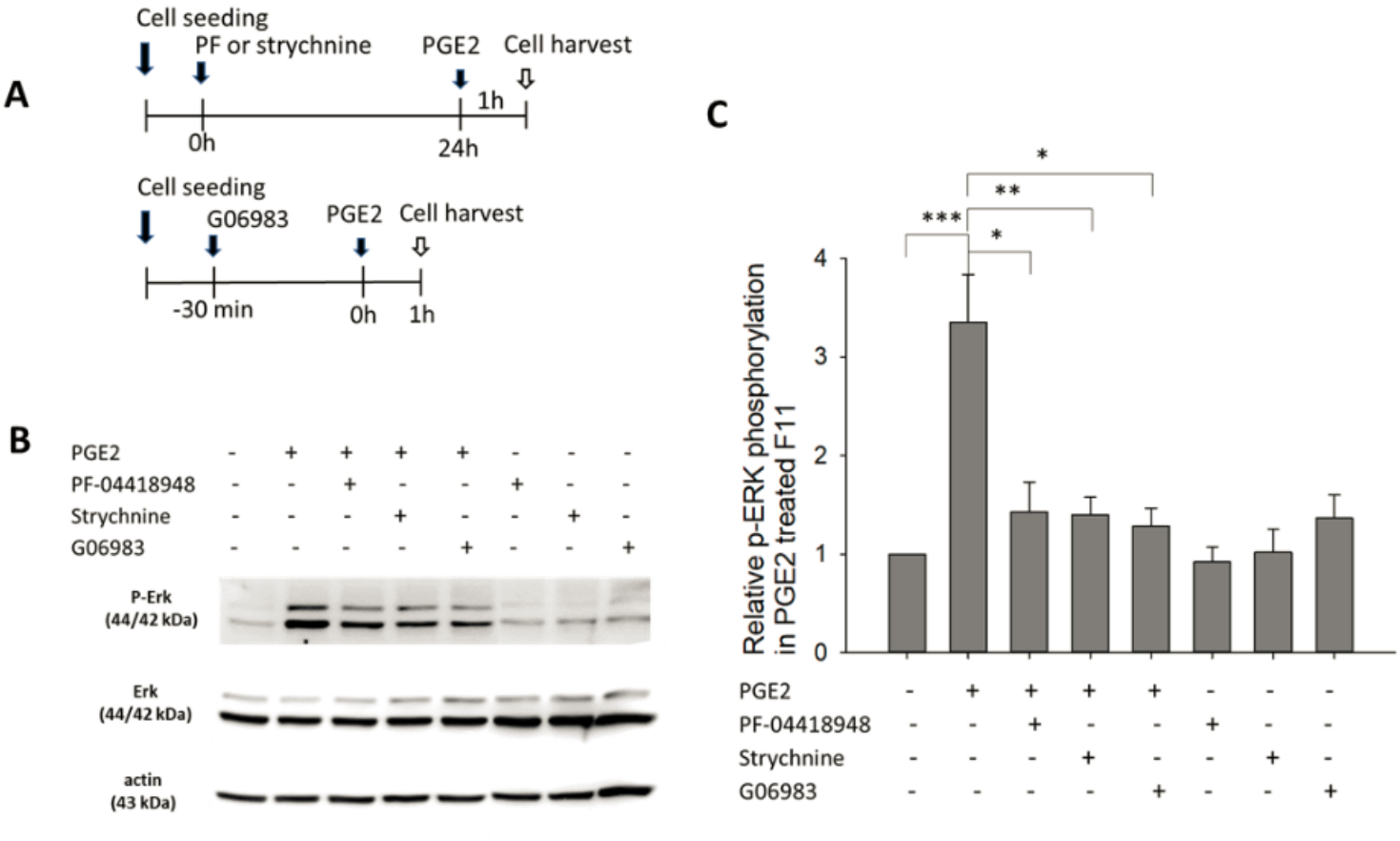
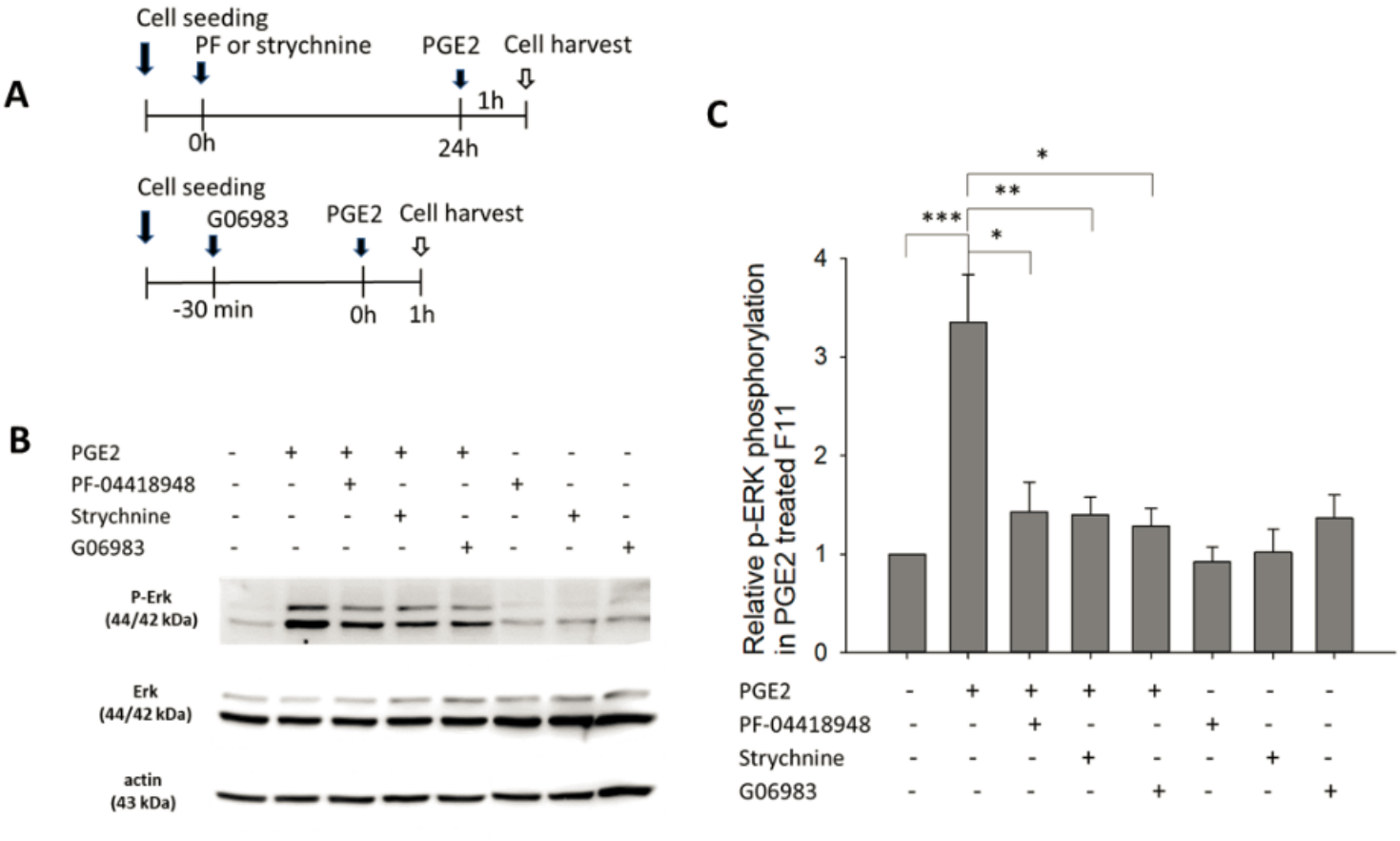


Figure 7

Effects of prostaglandin EP2-receptor antagonist (PF-04418948), glycine-receptor antagonist (strychnine), and PKC inhibitor (G06983) on PGE2-induced pERK phosphorylation. (A) F11 cells were seeded into a 6-well plate for 24 hours; PF-04418948 (10 μM) or strychnine (10 μM) was applied and incubated for 24 hours before PGE2 treatment. G06983 (3 μM) was applied 30 minutes before PGE2 treatment. One hour after PGE2 (100 μM) application, cells were harvested for protein extraction. (A) Western blotting of pERK phosphorylation, and (B) quantification of pERK phosphorylation in F11 cells. F11 cells without inhibitors or PGE2 treatment were used as a control. Data are presented from five independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001, one-way ANOVA.



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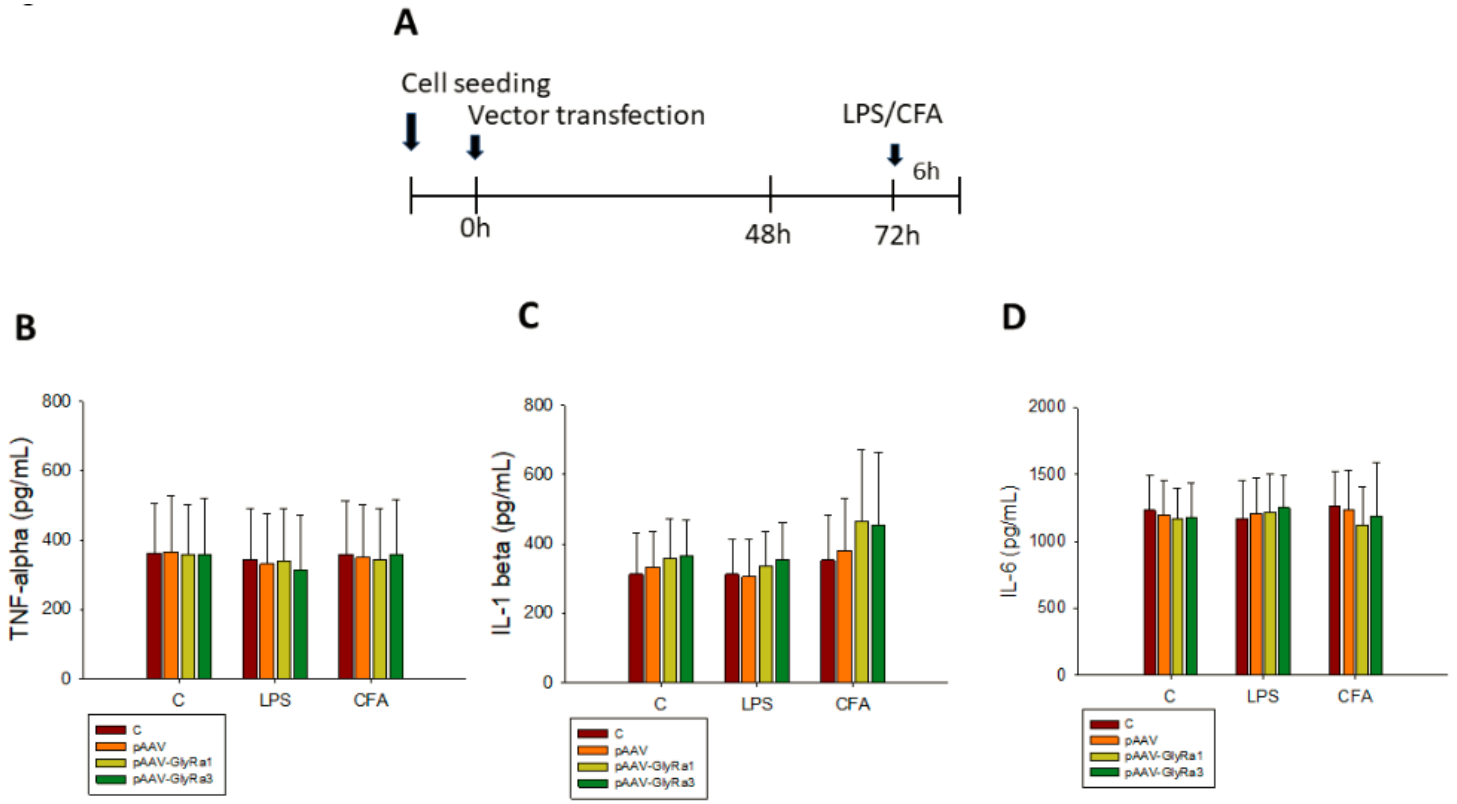
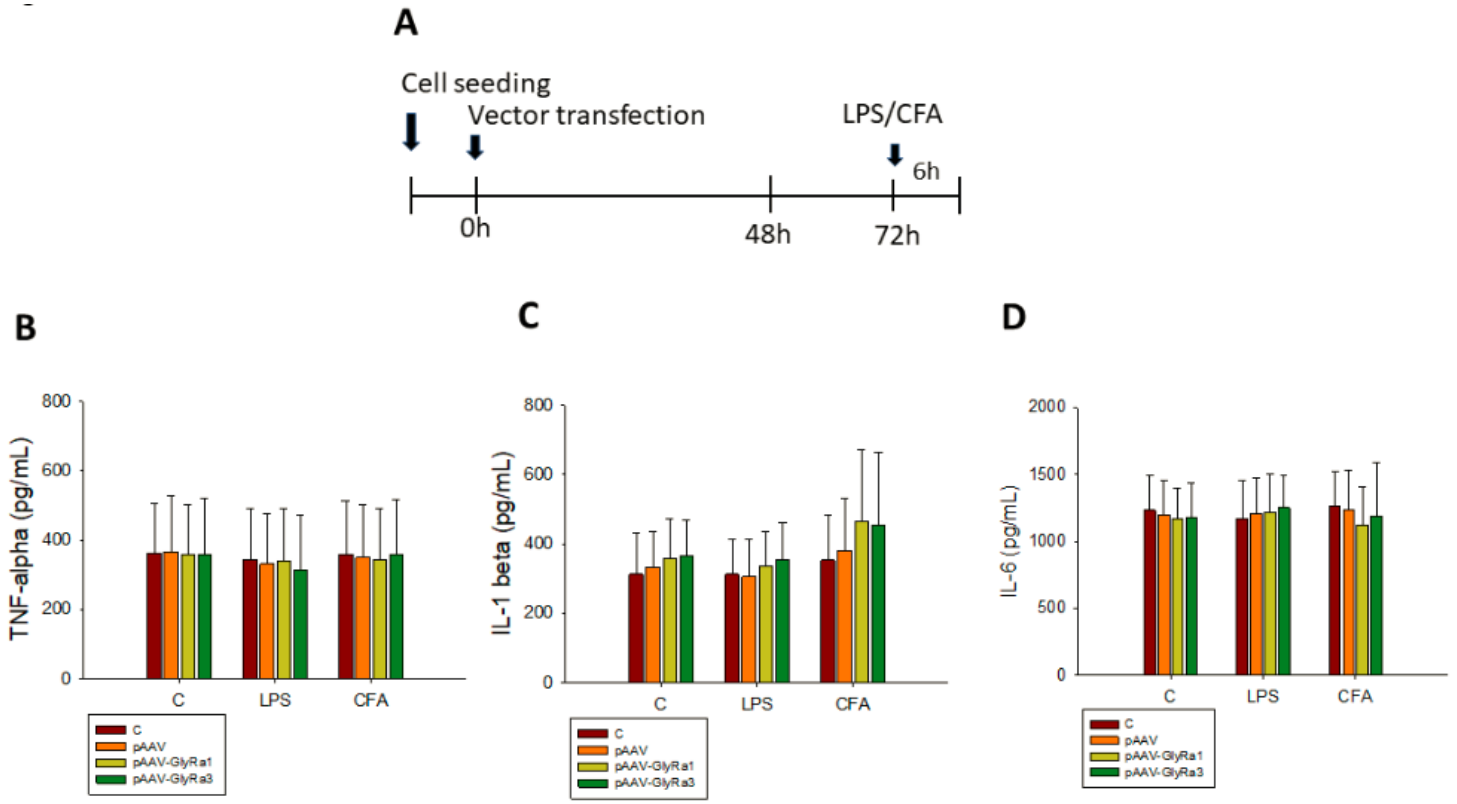


Figure 8

Level of cytokines expression was measured by ELISA. (A) Culturing F11 cells into the 6 well plate for 24 hours, pAAV, pAAV-GlyRα1 or pAAV-GlyRα3 was transfected respectively for 48 hours. The medium was changed into the serum free medium, 24 hours later, LPS (10 μM) or CFA (10 μM) was applied for 6 hours. Finally, the supernatant were collected for cytokines (B) TNF-α, (C) IL-1β and (D) IL-6 measurement in F11 cells by ELISA. The results are expressed as means ± SE, and the data shown represent independent experiments from three-five different experiments. Mann- Whitney U test was used.



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