Role of Glycine Receptor α3 in the Phosphorylation of Extracellular Signal-Regulated Kinase by Prostaglandin E2

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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 E₂ (PGE₂)-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 PGE₂-induced ERK1/2 phosphorylation in F11 cells was repressed by the expression of pAAV-GlyRα3 and administration of an EP₂ inhibitor (PF-04418948), GlyRαs antagonist (strychnine), and protein kinase C inhibitor (G06983).

Conclusions: PGE₂-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 PGE₂-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 E₂ (PGE₂) is an important mediator of the pathogenesis of inflammatory diseases, achieved through activation of EP₂ 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 PGE₂, while PKCε mediates the prolonged phase.[15–17] PGE₂ 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 PGE₂-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 PGE2-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 °C 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’-GGCGATATCTCACTTGGTGAGCTGTC-3’); primers for GlyRα3 (forward: 5’-ACAGCGGCCGCACCATGTACAGCTTCAACACTCTG-3’, reverse: 5’-GGCGATATCTTGAACACTTGGTGAGCTGTC-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'-GTAGTGCTTGGTGCACTA-3'; primers for GlyRα3 (forward: 5'-AAACACTACAATACAGGAAAGTTTAC-3', reverse: 5'-CAGTGCGATTACCCCGAAGGC-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'-GTAGTGCTTGGTGCACTA-3'; primers for GlyRα3 (forward: 5'-AAACACTACAATACAGGAAAGTTTAC-3', reverse: 5'-CAGTGCGATTACCCCGAAGGC-3') and DNA sequencing. The correct recombinant clones which containing pAAV-GlyRα1, or pAAV-GlyRα3 were store at -80°C 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 µg or 5 µg) 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 µg 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 37°C, 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 µg 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-GlyRa1 or pAAV-GlyRa3 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-GlyRa1 or pAAV-GlyRa3 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-1β, tumor necrosis factor (TNF)-α, and IL-6 by ELISA (R&D Inc., Minneapolis, MN, USA). F11 cells without pAAV, pAAV-GlyRa1, or pAAV-GlyRa3 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 PGE₂, 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 PGE₂ 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 PGE₂ 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 PGE₂ (100 ng) (Figs. 4B, C). Importantly, PGE₂ 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 PGE₂-induced ERK1/2 phosphorylation (Fig. 6A). We found that PGE₂ administration induced ERK1/2 phosphorylation; however, pre-transfected pAAV-GlyRα3 (but not pAAV-GlyRα1) significantly suppressed PGE₂-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-GlyR$_{\alpha 1}$, and pAAV-GlyR$_{\alpha 3}$ 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-$\alpha$ (Fig. 8B), IL-1$\beta$ (Fig. 8C), and IL-6 (Fig. 8D) were not induced in LPS/CFA-treated F11 cells, or those transfected with pAAV, pAAV-GlyR$_{\alpha 1}$, or pAAV-GlyR$_{\alpha 3}$.

Discussion

We found that pAAV/pAAV-GlyR$_{\alpha 1}$/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-GlyR$_{\alpha 3}$.

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-GlyR$_{\alpha 1}$/3 transfection, caused by the reagent. This indicates that pAAV/pAAV-GlyR$_{\alpha 1}$/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-GlyR$_{\alpha 3}$ transfection was caused by lipofectamine. Furthermore, our in vitro results indicated that exogenous pAAV-GlyR$_{\alpha 3}$ 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 GlyR$_{\alpha 1}$ subtype is associated with tumorigenesis and alcoholism,[28, 29] and the GlyR$_{\alpha 3}$ subtype is associated with inflammatory hyperalgesia.[1, 9, 12]
PKC-dependent phosphorylation of p38 and ERK have been reported.\cite{30} The phosphorylation of ERK occurs in a PKA-independent manner.\cite{31} Conversely, Chen et al. showed that PKA stimulated p38 and ERK phosphorylation in breast adipose fibroblasts.\cite{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.\cite{18}

Our study showed that pAAV-GlyR$\alpha$3 transfection or administration of glycine-receptor antagonist (strychnine) can downregulate PGE$_2$-induced ERK1/2 phosphorylation in F11 neuron cells. There are two possible explanations for this. First, ERK1/2 phosphorylation is also controlled by GlyRs through the PKA/PKC-dependent pathway.\cite{9, 26, 32} Second, the glycine-receptor structure changes during strychnine binding, as does the internal domain binding site of the PGE$_2$-dependent PKA/PKC pathway.\cite{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.\cite{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-$\alpha$\cite{35} and IL-6\cite{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.\cite{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.\cite{18, 27} Both LPS\cite{37–39} and CFA\cite{40, 41} are strong inflammatory mediators and can induce PGE$_2$ synthesis in animal models. Our results showed that the expression of TNF-$\alpha$, IL-1$\beta$, and IL-6 did not change in F11 cells treated by LPS/CFA or transfected with pAAV, pAAV-GlyR$\alpha$1, or pAAV-GlyR$\alpha$3. Hashemian et al. also used F11 neuron cells and found that LPS induced modest increases in IL-6 and COX2 expression, but did not induce significant TNF-$\alpha$ expression.\cite{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\cite{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-$\alpha$. 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-GlyR$\alpha$1/3 transfection does not induce cell cytotoxicity, ERK1/2 phosphorylation, or ATF-3 activation in neuron cells. Furthermore, pre-transfected pAAV-GlyR$\alpha$3 can significantly suppress PGE$_2$-induced ERK1/2 phosphorylation. We suggest that PGE$_2$-induced ERK1/2 phosphorylation can be modulated by GlyR$\alpha$3. pAAV/pAAV-GlyR$\alpha$1/3 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; PKC$\varepsilon$, 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-$\alpha$, tumor necrosis factor $\alpha$; 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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**Authors' contributions:** Conception and Design: HCW, ALK and LLC. Acquisition of Data: HCW, KIC and KYT. Analysis and Interpretation of Data: LLC. Drafting the Article: HCW and KIC. Critically Revising the Article: ALK and LLC. Reviewed submitted version of manuscript: HCW, KIC, KYT, ALK and LLC. Statistical analysis: LLC. Administrative / technical / material support: KIC and KYT. Study supervision: LLC. All authors read and approved the final manuscript.

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