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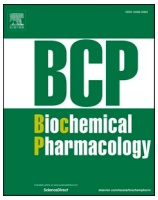

Activated microglia secretome and proinflammatory cytokines increase neuronal mu-opioid receptor signalling and expression

Javier Cuitavi [a,b], Pere Duart-Abadia [a,c,d], Julie Sanchez [e,f], Christian M. Sánchez-López [b,g], Jesús D. Lorente [b], Antonio Marcilla [b,g], Isabel Fariñas [a,c,d], Meritxell Canals [e,f,*], Lucía Hipólito [a,b,*]

[a] Instituto de Biotecnología y Biomedicina (BIOTECMED), University of Valencia, Burjassot, Spain
[b] Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Burjassot, Spain
[c] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), University of Valencia, Burjassot, Spain
[d] Departament of Cellular Biology, Functional Biology and Physical Anthropology, University of Valencia, Burjassot, Spain
[e] Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK
[f] Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, the Midlands, UK
[g] Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics UV-IIS La Fe, Valencia, Spain

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ABSTRACT

Due to its potential role in processes which rely on mu-opioid receptor function, investigating the relationship between Mu-Opioid receptors (MORs), neuroinflammation, and glial cells has gained momentum. Traditionally, MOR activation has been associated with immunosuppression, but recent findings suggest a more nuanced, bidirectional relationship with the immune system. To further investigate this relationship, herein, we investigated the role of the activated microglia secretome and proinflammatory cytokines in neuronal MOR expression and signalling. Our results show that both microglial secretome and specific cytokines increase neuronal MOR expression and enhance the [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-induced MOR activation. We also show that DAMGO-induced neuroinflammation increases neuronal MOR expression, activation, and regulation. Our findings suggest a feedback loop between microglial activation, cytokine release, and neuronal MOR dynamics. Future research should delve into the temporal dynamics and functional implications of this relationship, particularly concerning clinically relevant opioids like morphine and fentanyl and pain management.

Abbreviations: DAMGO, D-Ala2, N-MePhe4, Gly-ol]-enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DAPI, 4′,6-diamidino-2-fenilindol; BCA-1, B cell-Attracting Chemokine 1; BCA, Bicinchoninic Acid; BRET, Bioluminescence Resonance Energy Transfer; BSA, Bovine Serum Albumin; TARC, CC chemokine ligand 17; (C-C motif) ligand 1 (I309), Chemokine Chemokine (C-X-C motif) ligand 9 (MIG); CMV, Citomegalovirus; CSF, Colony Stimulating Factor; C5a, Complement component 5a; cDNA, complementary Deoxyribonucleic Acid; D2R, Dopamine Receptor 2; DMEM/F12, Dulbecco's modified Eagle's medium with Nutrient Mixture F12; DPBS, Dulbecco's Phosphate-Buffered Saline; EDTA, ethylenediaminetetraacetic acid; FMO, Fluorescence Minus One; FACS, Fluorescence-activated Cell Sorting; FBS, Foetal Bovine Serum; FCS, Foetal Calf Serum; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; HBSS, Hanks' Balanced Salt Solution; HRP, Horseradish peroxidase; I-TAC, IFN-inducible T cell α chemoattractant; IP10, IFNγ-induced protein 10; IFN, Interferon; IL, Interleukin; KC, Keratinocyte-derived Cytokine; LPS, Lipopolysaccharide; MIP, Macrophage Inflammatory Protein; TIMP1, Metalloprotease Inhibitor 1; MAC, minimum alveolar concentration; MPC, Mitochondrial Pyruvate Carrier; MCP, Monocyte Chemoattractant Protein; MORs, Mu-Opioid receptors; NLuc, Nano Luciferase; NLRs, Nod-like receptors; NAcC, Nucleus accumbens Core; PFA, Paraformaldehyde; PBS, Phosphate Buffered Saline; PDL, Poly-D-Lysine; ROS, reactive oxygen species; RT-qPCR, Real Time Quantitative Polymerase Chain Reaction; RANTES, Regulated on Activation Normal T-cell Expressed and Secreted; RLuc8, Renilla luciferase 8; RNA, Ribonucleic Acid; SDS-PAGE, Sodium Dodecyl Sulphatepolyacrilamide gel electrophoresis; siCAM1, Soluble Intercellular Adhesion Molecule-1; SEM, Standard Error of Mean; SDF-1, Stromal cell-derived factor 1; TBS-T, TBSTween; TLRs, Toll-like receptors; TGF-β2, Transforming growth factor-β2; TREM-1, Triggering Receptor Expressed on Myeloid cells 1; TBS, Tris-Buffered Saline; TNFα, tumour necrosis factor α; Vh, Vehicle.

* Corresponding authors at: Instituto de Biotecnología y Biomedicina (BIOTECMED), University of Valencia, Burjassot, Spain (L Hipólito); Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK (M Canals).
E-mail addresses: M.Canals@nottingham.ac.uk (M. Canals), lucia.hipolito@uv.es (L. Hipólito).

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1. Introduction

Mu-Opioid receptors (MORs) are metabotropic receptors, which pay a crucial role in many neurophysiological processes such as pain management, euphoria, sedation, miosis, addiction, truncation rigidity, nausea, and respiratory control, in the nervous system [1]. Interestingly, many of these processes present a strong inflammatory component within the central nervous system [2–5], suggesting that neuroinflammation in MOR-related processes might play an important role in opioid-induced behaviours. In fact, there is evidence that MORs activity is tightly associated with the immune system [4]. However, there is still some controversy regarding the nature of this relationship, which seems to be bidirectional.

Traditionally, MOR activation in the periphery has been regarded as immunosuppressive since there is previous evidence of its influence in the decrease in the activity of several immune cells. However, this seems to occur at a systemic level and but not centrally [4,6–9]. While this has raised concerns with regards to opioid-treated cancer patients, reports differ on the effect of MOR antagonists on tumour growth and cancer metastasis [10].

Contrarily, there is evidence that MOR within the central nervous system acts as a promoter of neuroinflammation [11–18,3,4]. The implications of MOR-mediated neuroinflammation raise intriguing questions about the intricate balance between immunosuppression and inflammatory responses, particularly in the context of neurological disorders and conditions affecting the central nervous system.

Additional evidence shows that proinflammatory cytokines such as interleukin (IL)1β, IL6 and tumour necrosis factor α (TNFα) increase the expression of the *OPRM1* gene in several cell types [19–22], including the astrocytoma cell line U97 MG, where the levels of both MOR messenger Ribonucleic Acid (RNA) and protein were upregulated upon treatment with IL1β [23]. Interestingly, Langsdorf and colleagues proposed that reactive oxygen species (ROS) also played a role in the regulation of *OPRM1* gene transcription in SH-SY5Y neuron-like cells [24]. Despite these reports, it is still unclear whether neuroinflammation modifies neuronal MOR signalling. Along these lines, some reports suggest that the activation of the canonical inflammatory pathway involving Toll-like and Nod-like receptors (TLRs and NLRs, respectively) and microglial MORs can contribute to opioid tolerance [25–28]. However, the role of neuroinflammation on MOR desensitisation, a key mechanism of opioid tolerance, has not been described yet.

The central hypothesis of the present work is that proinflammatory cytokines influence MOR expression and signalling. Therefore, herein, we investigated the relationship between microglia and MOR with a particular focus on how proinflammatory cytokines regulate neuronal MOR expression, activation, and trafficking, with the aim of understanding how alterations induced within neuroinflammatory processes can affect the action of this important therapeutic target.

2. Materials and methods

2.1. Animals

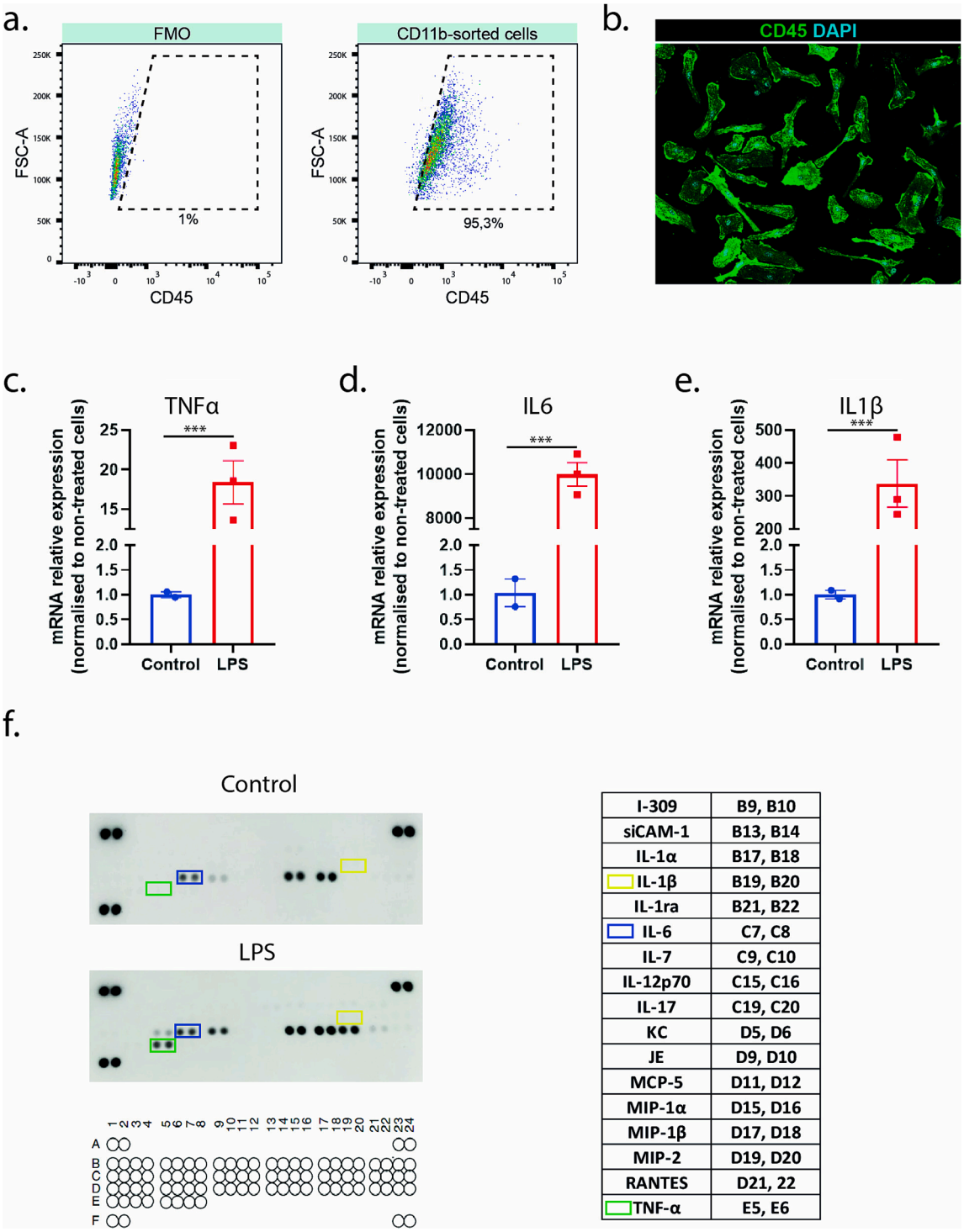
10 female and 10 male C57BL6/J mice 6–8 weeks old; Jackson Laboratories), and 5 males and 6 females Sprague Dawley rats (9–12 weeks old; Envigo®) were used. All the animals were kept in light/dark (12/12 h) controlled cycles, temperature 23 ± 1°C, and 60 % humidity. Animals were housed in standard plastic cages (mice: 42 × 26 × 14 cm^3; rats:42 × 27 × 18 cm^3) with food and tap water provided *ad libitum* throughout the experimental period. Animals were housed in the animal facilities of the SCSIE from the University of Valencia. Animal protocols followed in this work were approved by the Animal Care Committee of University of Valencia, and were strictly adhered to, in compliance with the EEC Council Directive 63/2010, Spanish laws (RD 53/2013) and animal protection policies.

2.2. Primary microglial cell cultures, treatment, and cytokines production assessment

Microglial cell cultures were performed as detailed by Bohlen and colleagues [29]. Briefly, male and female mouse brains were extracted, and the cerebellum and the olfactory bulbs were removed. Then the tissue was mixed and homogenised, first mechanically with a scalpel and then chemically with a gentleMACS™ Dissociator (Miltenyi Biotec). We mixed male and female brains in the same proportion to include both sexes in our study. After chemical digestion, tissue was filtered with 40 μm filters. This was followed by an immunoprecipitation step using CD11b MicroBeads and MS Columns from Miltenyi Biotec to discard non-expressing CD11b cells, CD11b being a marker of microglia. Finally, microglial cells were counted and cultured in 6-well plates 50,000 cells/plate) coated with Poly-D-Lysine (PDL). Cells were maintained at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 in Dulbecco's modified Eagle's medium with Nutrient Mixture F12 (DMEM/F12) without phenol red supplemented with 1 % Penicillin-Streptomycin, 1 % L-Glutamine, N-Acetylcysteine (5 μg/mL), Sodium Selenite (100 pg/mL), Apo-Transferrin (100 μg/mL), Colony Stimulating Factor (CSF)1; 10 pg/mL), Transforming growth factor-β2 (TGF-β2; 2 pg/mL), Heparan Sulphate (1 μg/mL).

The purity of the isolation was assessed by Fluorescence-activated Cell Sorting (FACS) before the cell culture (Fig. 1a). Briefly, cells were incubated with 100 μl of the fluorescent-labeled primary antibody BUV395 Rat Anti-Mouse CD45 (BD Bioscience) in flow cytometry blocking buffer (0.1 % Glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 % Bovine Serum Albumin (BSA) in Hanks' Balanced Salt Solution (HBSS)) on ice for 30 min. After washing, labeled samples were resuspended in 0.5 ml of blocking buffer for analysis in an LSR-Fortessa cytometer (350, 405, 488, 561, and 640 nm lasers, Becton Dickinson) using FACSDiva software (v8.0.2, BD). 4′,6-diamidino-2-fenilindol (DAPI; 0.1 μg/ml) was added to exclude dead cells from the analysis. Moreover, immunocytochemistry of fluorescence was used after the onset of the culture to further test microglia purity (Fig. 1b). Microglial cells were fixed with 2 % paraformaldehyde (PFA) for 20 min. Before immunolabeling, unspecific binding of antibodies was minimized by incubating the cells in blocking buffer (10 % Foetal Bovine Serum (FBS), 1 % glycine, 0.1 M Phosphate Buffered Saline (PBS)) for 30 min. Subsequently, samples were incubated with rat anti-CD45 primary antibody (1:400, BD) in blocking buffer overnight at 4 °C and then with the Alexa 488-donkey anti-rat fluorescent-labeled secondary antibody (1:800; Molecular Probes) diluted in blocking buffer for 1 h at room temperature. Finally, cells were washed with PBS, and nuclei were counterstained with 1 mg/ml DAPI for 5 min. Images were acquired using a Nikon ECLIPSE Ni-U microscope (Nikon) with a Zyla 4.2 sCMOS camera (Andor).

A week after isolation, cells were treated with either 10 ng/mL lipopolysaccharide (LPS) for 24 h or the same relative volume of sterile water (control). The LPS dose chosen was sufficient to produce inflammation without being damaging to the cells [30]. Then, the cell culture medium was collected, filtered with 0.22 μm filters and profiled using a Proteome Profiler™ Mouse Cytokine Array Panel A (R&D systems) following the manufacturer's guidelines for the semiquantitative assessment of cytokine production. Cells were also collected for the assessment of the cytokine production through real time quantitative polymerase chain reaction (RT-qPCR). For this, microglial cells were lysed with RLT plus buffer and the RNA was extracted with the RNeasy Plus Micro Kit (QIAGEN) following the manufacturer's instructions. The RNA obtained was then quantified using the Qubit_RNA HS Assay Kit (Thermo Fisher) in a Qubit Fluorometer (Thermo Fisher). Then RNA was retrotranscribed to complementary Deoxyribonucleic Acid (cDNA) using the PrimeScriptTM RT-PCR Kit (Clontech) according to the manufacturer's instructions. Gene expression analysis was assessed using 10–20 ng of cDNA, specific Taqman probes (Applied Biosystems) and the



I-309	B9, B10
siCAM-1	B13, B14
IL-1α	B17, B18
IL-1β	B19, B20
IL-1ra	B21, B22
IL-6	C7, C8
IL-7	C9, C10
IL-12p70	C15, C16
IL-17	C19, C20
KC	D5, D6
JE	D9, D10
MCP-5	D11, D12
MIP-1α	D15, D16
MIP-1β	D17, D18
MIP-2	D19, D20
RANTES	D21, 22
TNF-α	E5, E6

Fig. 1. Assessment of microglial culture purity and cytokine production in microglia secretome. a: FACS dot plot depicts the CD11b + fraction, highlighting over 95 % of cells as CD45+, confirming the high purity of microglial cultures (right panel). The left panel illustrates the CD45 intensity of the Fluorescence Minus One (FMO) control, used to establish the gating for the CD45 signal. b: Immunofluorescence confirms widespread CD45 expression in cultured cells, further affirming microglial purity. DAPI (cyan) was employed for nuclear counterstaining. c-e: RT-qPCR (n = 2–3/group; c: TNFα, d: IL6, e: IL1β). f: Cytokine Array Panel image and template. The positions within the membranes of the cytokines that qualitatively increase their expression after the treatment with LPS are indicated in the table. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.005 (T-test for independent samples). Lipopolysaccharide (LPS).

Premix Ex TaqTM (Probe qPCR) Kit (Clontech). RT-qPCR was performed in a Step One Plus PCR device (Applied Biosystems). The expression level of each gene was obtained by relative quantification (2(–ΔΔCt)) using constitutive expression of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and 18S genes as housekeeping endogenous controls.

Taqman probes:

- 18S TaqMan_Probe Thermo Fisher Cat#4331182; Hs99999901_s1
- GAPDH TaqMan_Probe Thermo Fisher Cat#4331182; Mm99999915_g1
- TNFα TaqMan_Probe Thermo Fisher Cat#4331182; Mm00443258_m1
- IL1β TaqMan_Probe Thermo Fisher Cat#4331182; Mm00434228_m1

- IL6 TaqMan_Probe Thermo Fisher Cat#4331182; Mm00446190_m1

2.3. Protein fraction isolation

Cell culture media from primary microglia cell cultures underwent a process of separation by centrifuging twice and collecting the supernatant (3000xg for 15 min, and 16000xg for 20 min). Samples were then filtered by using 0.22 μm filters and concentrated with an Amicon® Ultra-4 tube (Merck). 12 fractions of 100 μL were obtained by using a 20 μm pore size column (Merck) loaded with 1 mL of stacked Sepharose CL-2B (Sigma Aldrich), using PBS as elution buffer. The protein concentration of every fraction was measured using a Micro Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific). Fractions 9–12 were pooled as the most enriched protein fractions. Finally, samples were taken to the pre-concentration volume so that all the proteins returned to their original concentrations.

2.4. Microdialysis

Rats underwent a microdialysis experiment to obtain *in vivo* samples of [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-induced extracellular release of cytokines. Surgeries were performed under isoflurane anesthesia (1.5–2 minimum alveolar concentration (MAC) in aseptic conditions. Rats were used for these experiments to obtain an amount of Nucleus accumbens Core (NAcC) dialysate sufficient to perform cell-based assays. Rats were stereotaxically implanted (Stoelting) with bilateral vertical guide cannulae (CMA 12) into the nucleus accumbens core (NacC; Males – anteroposterior = 1.4 mm, mediolateral = 1.5 mm, and dorsoventral = 5.8 mm; Females – anteroposterior = 1.2 mm, mediolateral = 1.4 mm and dorsoventral = 5.6 mm). 48 h after surgery animals were placed in Plexiglas bowls and concentric-style microdialysis probes containing 2 mm of active membrane (CMA 12 HighCo; molecular *cutoff* 100,000 Da) were introduced through the cannulae, extending 3.0 mm below the tip of the cannulae. Then, artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$ at pH = 6) containing 0.5 mg/ml BSA was perfused into the probe through a push–pull system, which consisted of an inlet tubing attached to a 2.5-mL syringe (Hamilton) mounted on a syringe pump (Harvard instruments) and an outlet tubing connected to a peristaltic pump (Ismatec®). Pumps were set at 2.2 μL/min. After a 1 h wash of the system, 5 dialysates were collected every 100 min. After the collection of the first dialysate a treatment with 1 μM DAMGO was administered by retrodialysis for 20 min. These dialysates were analysed in Cuitavi and colleagues [3] where the production of proinflammatory cytokines was characterised by flow cytometry (Fortessa) using a customised multiplex approach (LEGENDplex™, BioLegend®). For this study, we used two dialysates: the baseline sample collected prior to DAMGO administration (−100 to 0 min dialysate), and a post-treatment sample (100 to 200 min dialysate). To ensure that DAMGO had been completely washed out after pretreatment, we discarded the immediate dialysate subsequent to DAMGO retrodialysis application (0 to 100 min dialysate).

2.5. SH-SY5Y cell cultures and treatments

SH-SY5Y (ATCC HTB-11) cells were maintained in DMEM/F12 supplemented with 10 % heat inactivated Foetal Calf Serum (FCS) and 2 mM L-Glutamine at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Undifferentiated cells underwent transient transfections using FuGENE® HD Transfetion Reagent (Promega) at a DNA:FuGENE® ratio of 1:3 according to the manufacturer's guidelines. Cells were transfected with different amounts of DNA depending on the experiment:

- MOR bioluminescence for MOR expression: 1 μg of FLAG-mMOR-Nluc, and 4 μg of pcDNA3.1. 1 μg of D2-Nluc was used as control.
- βγ release for G protein activation: 2 μg of $G\alpha_{i2}$, 1 μg of Venus155-239–$G\beta_1$, 1 μg of Venus1-155–$G\gamma_2$, 1 μg of masGRK3ct-Rluc8, and 1 μg of SNAP-mMOR.
- β-Arrestin 2 recruitment: 1 μg of FLAG-mMOR-Nluc, 1 μg of GRK2, and 4 μg of β-Arrestin 2–Venus.

Cells were replated into PDL–coated 96-well plates 24 h after transfection and allowed to adhere for 4 h. Then the medium was replaced by DMEM/F12 supplemented with and 2 mM L-Glutamine without FCS, which contains cytokines and other inflammatory mediators, and then cells were treated for 24 h with:

- Medium from microglia treated with either vehicle or LPS.
- Protein fraction of the medium coming from microglia treated with either vehicle or LPS.
- TNFα (500 pg/mL), IL6 (1800 pg/mL), IL1β (60 pg/mL) or vehicle (reported to be present in the media of a primary microglial cell culture after a 24 h treatment with 10 ng/mL LPS [31]).
- The rat brain dialysate pre- and post- DAMGO treatment (mixed male and female dialysates since we did not observe sex-dependent significant differences in the release of proinflammatory cytokines [3]).

Of note, the morphology of the cells was monitored throughout the experiment showing no differentiation or evident changes within the incubation period.

2.6. MOR expression by bioluminescence

Cells transfected with FLAG-mMOR-Nluc underwent the treatments stated above. After 24 h incubation, the cell permeable substrate Coelenterazine h (NanoLight Technologies, AZ) was added at a final concentration of 5 μM. Luminescence was measured immediately in a PHERAstar Omega plate reader (BMG LABTECH, optic module, 475 ± 30). This experiment was performed three times in duplicate. Data is expressed as percentage of the vehicle-treated group. Cells transfected with D2-Nluc were used as a negative control.

2.7. MOR expression by western blot

Cells treated with activated medium from microglia were harvested after 24 h of incubation. Briefly, cells were washed with cold PBS. Then, 200 μL of cold lysis buffer (1 % IGEPAL CA-630, 20 mMTris-HCl pH 8,130MNaCl, 10mMNaF and 1 % proteases inhibitor) were added and cells were collected by scratching. Samples were transferred to a tube and were left in ice for 30 min. During that time, samples were vortexed every 10 min for 15 s. Then, samples were sonicated three times for 30 s with breaks of 30 s in between. Finally, samples were centrifugated at 13,000 x g for 10 min at 4 °C and the supernatant was collected. The concentration of the lysates was determined by using a Bradford protein assay kit (Bio-Rad).

1-mm acrylamide gels at 10 % with 15 wells were used for the electrophoresis. We mixed 20 μg of total protein, loading buffer (350 mM Tris pH 6.8, 30 % glycerol, 30 % mercaptoethanol, 100 g/L sodium dodecyl sulphate and 200 mg/L bromophenol blue) and water to obtain the same total protein and volume per sample. Then, samples were heated at 70 ∘C for 20 min. We used the Bio-Rad Mini-PROTEAN buffer system (6 g/L Trizma base, 2.88 g/L glycine and 20 g/L sodium dodecyl sulphate) to perform the electrophoresis at 120 V for 80–90 min. Once, the proteins were separated by sodium dodecyl sulphatepolyacrilamide gel electrophoresis (SDS-PAGE), we transferred it to nitrocellulose membranes (Bio-Rad) with the appropriate buffer (3 g/L Trizma base, 1.44 g/L glycine and 20 % methanol) and a semidry system (Bio-Rad Trans-Blot TurboTM) for 25 min at 25 V buffer. Then, membranes were blocked using 5 % BSA in Tris-Buffered Saline (TBS)Tween 20 (TBS-T) 0.1 % (20 mM Tris and 500 mM NaCl pH 7.5) for 1 h, and just before they were incubated with the corresponding primary antibody overnight

at 4 ∘C.; rabbit IgG anti-MOR (1:1000, Abcam) or rabbit anti-GAPDH (1:2000, Invitrogen). After that, membranes were washed 3 times with TBS-T 0.1 % and were incubated for an hour at room temperature with the secondary antibody goat anti-Rabbit IgG-HRP (1:2000, Invitrogen). Finally, we developed the membranes with chemiluminescence using CheLuminate-Horseradish peroxidase (HRP) PicoDetect (Panreac, Barcelona, Spain), and images were captured with the ChemiDoc XRS1 System (Bio-Rad) and quantified using ImageJ software. The grey intensity of the bands was normalised dividing the values of the protein of interest by the GAPDH values. We repeated this experiment four times in duplicate.

2.8. Bioluminescence resonance energy transfer (BRET)

βγ release and β-Arrestin 2 recruitment were performed 24 h after the treatments in cells transfected as described above. Cells are taken out of the incubator and washed with Dulbecco's Phosphate-Buffered Saline (DPBS). Then, they were incubated for 30 min with 5 mM glucose in DPBS at 37 °C. The cell permeable luciferase substrate Coelenterazine h (NanoLight Technologies, AZ) was used at a final concentration of 5 μM and after its addition cells were incubated for 5 min in the dark at 37 °C. Vehicle and 10 μM DAMGO were added 10 min before dual fluorescence/luminescence measurement in a PHERAstar Omega plate reader (BMG LABTECH). The BRET signal was calculated as the ratio of light emitted by Venus at 530 nm (optic module 535 ± 30) over the light emitted by NLuc or Rluc8 at 430 nm (optic module, 475 ± 30). We repeated BRET experiments three times in duplicate. A fold-over basal ratio was obtained by dividing DAMGO-treated BRET ratio by the vehicle-treated BRET ratio.

2.9. Statistical analysis

Results are shown as mean ± Standard Error of the Mean (SEM). To perform the statistical analysis, the 26.0 version of the SPSS program was used. The Kolmogorov–Smirnov test and Levene's test were performed to assess the normality and the homogeneity of the variance of the data. For the RT-qPCR analysis and the experiments where the treatments were either microglial cell media, the protein fraction of that media, or dialysates a T-test for independent samples was used. When cells were treated with proinflammatory cytokines a one-way ANOVA, followed by either Bonferroni or Games-Howell when appropriate, was used. In all the statistical tests, a 95 % confidence level was set.

3. Results

3.1. Microglial control over neuronal MOR expression and signalling

3.1.1. Assessment of the production of proinflammatory mediators by microglia

The production and release of proinflammatory mediators by microglial primary cell cultures (Fig. 1 a,b) treated with either vehicle or LPS (10 ng/mL) for 24 h was assessed by RT-qPCR and proteome cytokine arrays (Fig. 1 c-e). The mRNA levels of three proinflammatory cytokines (TNFα, IL6, and IL1β) were measured by RT-qPCR. The mRNA levels for these three cytokines were significantly higher in the LPS-treated cells than in the vehicle-treated cells (Fig. 1c, TNFα, $p = 0.0001$; Fig. 1d, IL6, $p = 0.0001$; Fig. 1e, IL1β, $p = 0.0001$). On the other hand, the proteome cytokine array revealed the release of several proinflammatory mediators upon LPS treatment (Fig. 1f). Indeed, this qualitative analysis showed increased content of Chemokine (C-C motif) ligand 1 (I309), Soluble Intercellular Adhesion Molecule-1 (siCAM1), IL1α, IL1β, IL1ra, IL6, IL7, IL17, Keratinocyte-derived Cytokine (KC), Mitochondrial Pyruvate Carrier (MPC)1, MCP5, Macrophage Inflammatory Protein (MIP)1α, MIP1β, MIP2, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), and TNFα in the LPS-treated microglia secretome in comparison to the vehicle-treated one. However, we also detected unchanged levels of some inflammatory mediators such as B cell-Attracting Chemokine 1 (BCA-1), Complement component 5a (C5a), granulocyte CSF, Granulocyte-Macrophage CSF, eotaxin, interferon (IFN)γ, IL2, IL3, IL4, IL5, IL10, IL12, IL13, IL16, IL23, IL27, IFNγ-induced protein 10 (IP10), IFN-inducible T cell α chemoattractant (I-TAC), Macrophage CSF, Monocyte Chemoattractant Protein (MCP)-1, MCP-5, Chemokine (C-X-C motif) ligand 9 (MIG), Stromal cell-derived factor 1 (SDF-1), CC chemokine ligand 17 (TARC), Metalloprotease Inhibitor 1 (TIMP1), and Triggering Receptor Expressed on Myeloid cells 1 (TREM-1).

3.1.2. Detection of MOR expression upon treatment with proinflammatory mediators

The bioluminescence corresponding to the Nluc tag fused to the C-terminus of a MOR construct expressed in neuronal-like SH-SY5Y cells was used to determine the expression levels of MOR protein when cells were treated with activated microglial secretome, the protein fraction of that secretome or proinflammatory cytokines for 24 h (Fig. 2). Interestingly, the expression of MOR protein was significantly increased when SH-SY5Y cells were exposed to all cytokine-enriched media (Fig. 2a, Microglial secretome ($p = 0.023$), Fig. 2b, Protein fraction ($p = 0.033$) and Fig. 2c, cytokines $F(3,11) = 50.714$, $p = 0.0001$, Games-Howell Vh-TNFα $p = 0.019$, Vh-IL6 $p = 0.026$, Vh-IL1β $p = 0.037$). To rule out that such increases were due to effects on the activity of the Citomegalovirus (CMV) promoter of the pcDNA3.1 plasmid carrying the tagged-MOR cDNA, we transfected the cells with a Dopamine Receptor 2 (D2R)-Nluc construct in the same pcDNA3.1 plasmid. None of the treatments did alter D2R expression on SH-SY5Y cells as shown in Fig. 2d-f (Microglial secretome, $p = 0.181$; Protein fraction, $p = 0.725$; Cytokines, $F(3,11) = 3.480$, $p = 0.070$). In addition, these changes in MOR expression were confirmed by western blot in SH-SY5Y cells detecting increased endogenous MOR in cells treated with activated microglial secretome (Fig. 2g, $p = 0.040$).

3.1.3. Measurement of G protein activation and β-arrestin 2 recruitment by MOR upon treatment with proinflammatory mediators

To assess MOR-induced G protein activation, a βγ release BRET assay was performed in transfected SH-SY5Y cells [32]. In this assay, activation of MOR by 10 μM DAMGO for 10 min is detected as the release of the βγ subunits from the activated Gαi subunit of the G protein. The released βγ subunit has high affinity for the lipid-modified reporter peptide GRK3ct (masGRK3ct-Nluc) and such interaction is measured through BRET. We observed that cells exposed to media obtained from LPS-treated microglial cells or to the protein fraction of those media had a significantly higher DAMGO-induced G protein activation than those exposed to medium or protein recovered from vehicle-treated microglia (Fig. 3a-c). The three tested cytokines had a similar inducing effect (Fig. 3a-c) ($p = 0.039$, $p = 0.005$, and one-way ANOVA $F(3,11) = 28.517$, $p = 0.0001$; Bonferroni multiple comparisons test Vh-TNFα $p = 0.001$, Vh-IL6 $p = 0.001$ Vh-IL1β $p = 0.0001$, respectively) (Fig. 3a-c).

Upon activation, phosphorylated MORs recruit β-arrestins to mediate receptor internalisation. We thus assessed whether the increases in G protein activation were also manifested when β-Arrestin 2 recruitment to the MOR was monitored [32]. For this, the BRET between β-Arrestin 2-Venus and MOR-Nluc was measured upon activation of the MOR with 10 μM DAMGO for 10 min. Fig. 3d-f show how, although β-Arrestin 2 was being recruited upon activation of the receptor with DAMGO, no differences were found between groups in any of our analysis for microglial secretome, protein fraction of the secretome and cytokines (Microglial secretome, $p = 0.799$; Protein fraction, $p = 0.838$; Cytokines, $F(3,11) = 0.926$, $p = 0.471$).

3.2. Activation of MOR on rat NAcC releases factors that control MOR expression and signalling in neuronal cells

Alongside other authors [11–18,33,34], we have previously

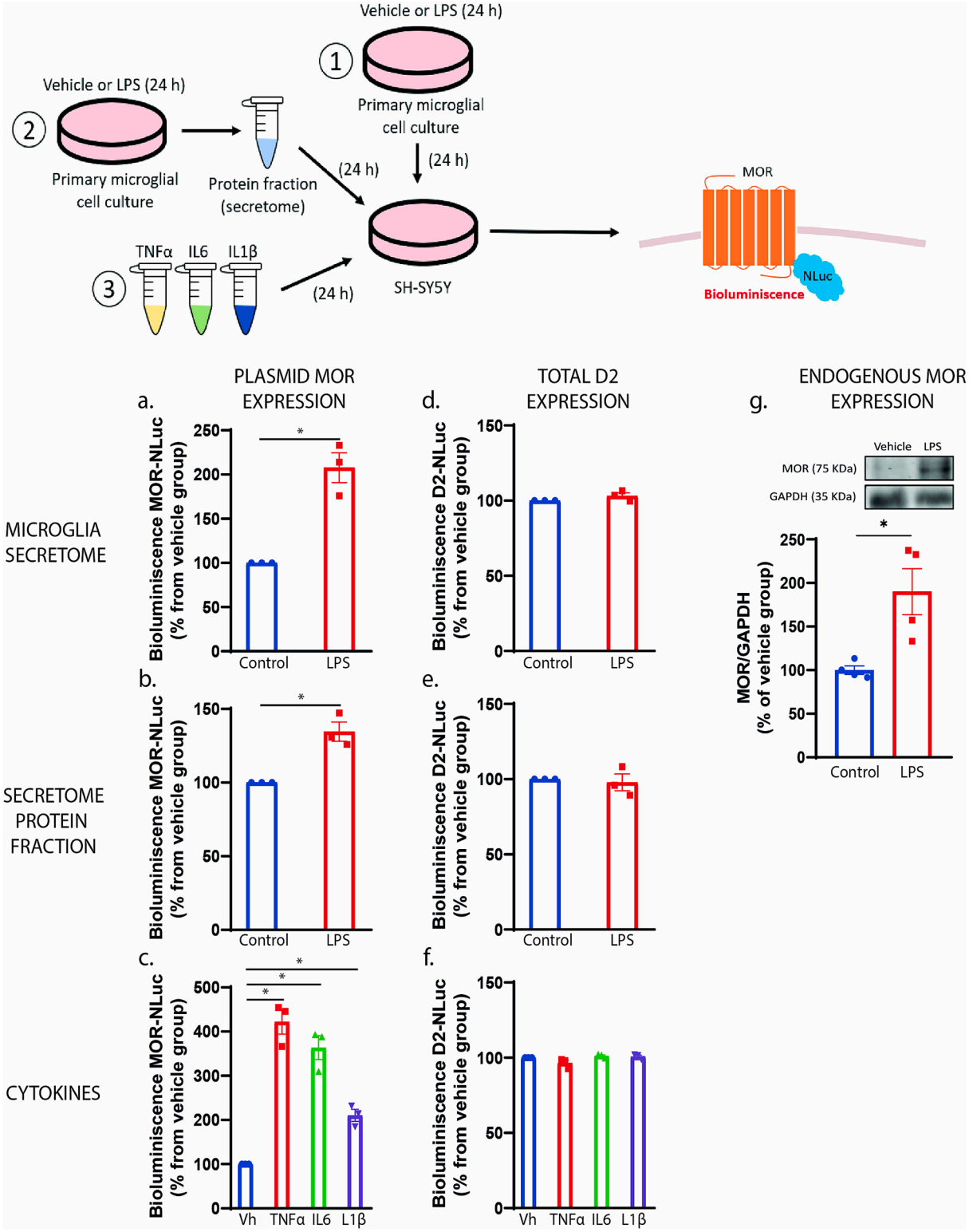


Fig. 2. MOR and D2R protein expression in SH-SY5Y cells treated with microglial secretome, its protein fraction, or proinflammatory cytokines. a-c: MOR (n = 3/group; a: secretome, b: protein fraction, c: cytokines). d-f: D2R (n = 3/group; d: secretome, e: protein fraction, f: cytokines). Data are expressed as mean ± SEM. *p < 0.05 (T-test for independent samples or one-way ANOVA followed by Games-Howell when appropriate). Lipopolysaccharide (LPS), mu-opioid receptor (MOR), dopamine D2 receptor (D2R), Nano Luciferase (NLuc), vehicle (Vh).

demonstrated that MOR activation can trigger neuroinflammation *in vivo* [3]. Therefore, we assessed whether neuroinflammatory mediators released upon MOR pharmacological activation can also regulate neuronal MOR expression and signalling. To do so, we carried out a microdialysis experiment in rats in which we obtained dialysates from NAcC every 100 min. The first dialysate was used as baseline whereas the second dialysate (200–300 min) after a retrodialysis administration of 1 μM DAMGO at a 2.2 μL/min flow rate for 20 min was used as the cytokine-enriched dialysate (see Cuitavi et al., 2023b for more details). Fig. 4 shows how treatment of SH-SY5Y with dialysates from *in vivo*

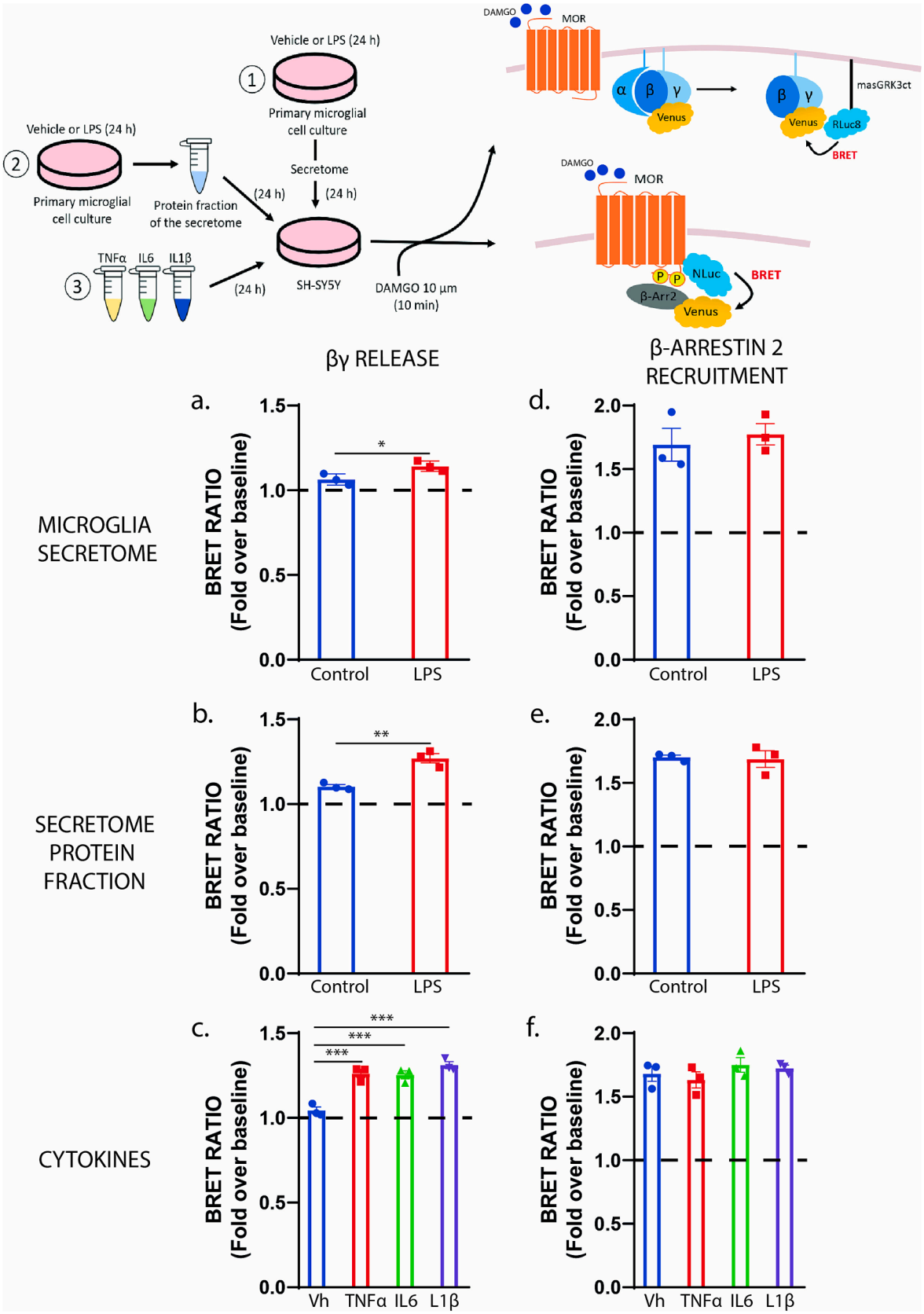


Fig. 3. G protein activation and β-arrestin 2 recruitment to MOR in cells treated with microglial secretome, its protein fraction, or proinflammatory cytokines. a-c: βγ release (n = 3/group; a: secretome, b: protein fraction, c: cytokines). d-f: β-arrestin 2 recruitment (n = 3/group; d: secretome, e: protein fraction, f: cytokines). Dotted lines indicate baseline. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005 (T-test for independent samples or one-way ANOVA followed by Bonferroni when appropriate). Bioluminescence resonance energy transfer (BRET), lipopolysaccharide (LPS), mu-opioid receptor (MOR), nano luciferase (NLuc), Renilla luciferase 8 (RLuc8), β-arrestin 2 (β-Arr2), vehicle (Vh), [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO).

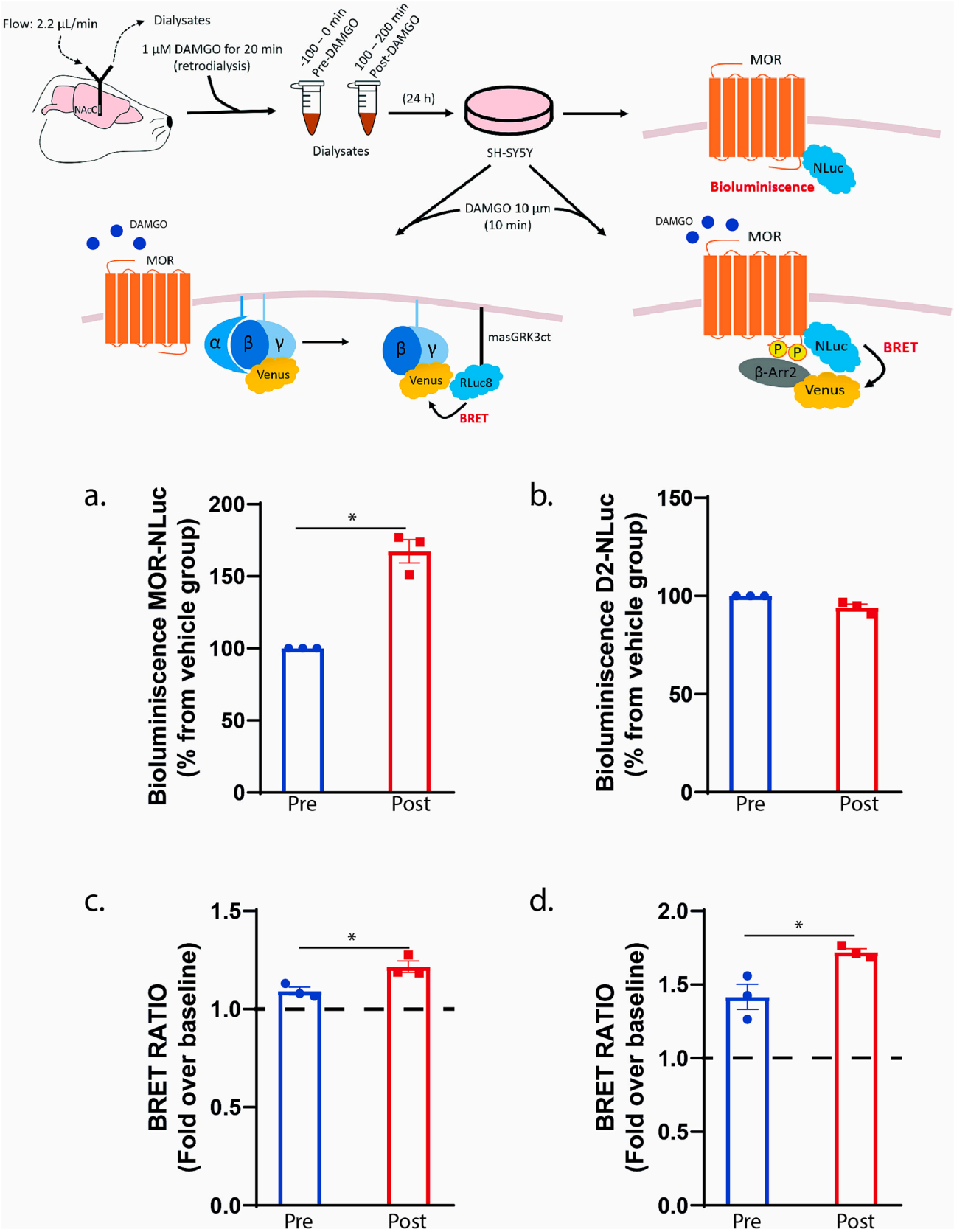


Fig. 4. MOR and D2R protein expression, G protein activation, and β-arrestin 2 recruitment in cells treated with DAMGO-induced cytokines-enriched dialysates from rat NAcC. a: MOR protein expression (n = 3/group), b: D2R protein expression (n = 3/group), c: βγ release(n = 3/group), d: β-arrestin 2 recruitment (n = 3/group). Data are expressed as mean ± SEM. *p < 0.05 (T-test for independent samples) Bioluminescence resonance energy transfer (BRET), mu-opioid receptor (MOR), dopamine D2 receptor (D2R), nano luciferase (NLuc), Renilla luciferase 8 (RLuc8), β-arrestin 2 (β-Arr2), vh (vehicle), [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), nucleus accumbens core (NAcC).

DAMGO-induced neuroinflammation significantly increased MOR protein levels (Fig. 4a, $p = 0.014$) without altering D2 levels (Fig. 4b, $p = 0.080$). Moreover, dialysates enriched with DAMGO-induced cytokines significantly increased MOR-mediated βγ release when compared to cells treated dialysates obtained prior to the administration of DAMGO to the rats (Fig. 4c, $p = 0.025$). Interestingly, cells treated with dialysates enriched with DAMGO-induced cytokines show significant higher levels of β-Arrestin 2 recruitment than when treated with pre-DAMGO-exposed dialysates (Fig. 4d, $p = 0.026$), which did not occur in the experiments involving microglial cells *in vitro* (Fig. 3).

4. Discussion

Here, we present how the secretome of LPS-activated microglial cells can alter MOR expression and signalling in neuron-like cells and how cytokines IL1β, TNFα, and IL6 exert the same effects as the microglial secretome. Moreover, we have previously demonstrated that MOR pharmacological activation in the mesocorticolimbic system leads to elevated proinflammatory cytokines release and microgliosis [3]. Interestingly, in the present paper we show how dialysates enriched in DAMGO-induced cytokines also increase MOR expression and signalling, which hints the existence of a positive feedback in MOR activation. Altogether our results and previous published data support that glial MORs might impact on neuronal MORs functioning through neuroimmune events.

Several reports have shown an increase in the expression of the MOR messenger RNA induced by proinflammatory cytokines in different cell types [19–22]. Of these, only Börner and colleagues used neuron-like cells (SH-SY5Y) to show an IL6-induced increase in the *OPRM1* expression [22]. Langsdorf and colleagues, using an approach similar to the one described here, showed that the medium from macrophage-like cells activated with LPS also increased *OPRM1* expression in SH-SY5Y cells [24]. Our results confirm the role of TNFα, IL6 and IL1β as inducers of MOR expression in SH-SY5Y cells. Furthermore, our data suggest that the upregulation occurs at the messenger RNA level, given that MOR is expressed from an exogenous plasmid whose promoter should not be affected by proinflammatory cytokine exposure, indicating that the observed changes likely occur after transcription. In addition, we report that LPS-induced microglial activation can increase MOR expression in neuron-like cells through the release of proteins, including but not limited to TNFα, IL6, and IL1β. Our results also show that microglial cytokines induced an increase in the activity of MORs in the SH-SY5Y cells. This is likely a direct consequence of the increase in MOR protein levels, although further concentration–response curves of MOR should confirm whether the effect observed correlate with increased potency opioid agonists.

Neuroinflammation has traditionally been perceived as an inducer of opioid tolerance [25–27,35]. In fact, there is evidence that neuroinflammation can decrease the analgesic effect of opioid agonists [36]. This suggests that neuroinflammatory mediators and cytokines could modulate opioid receptor sensitisation by influencing their trafficking and, therefore, membrane density. Of note, in our experiments we fail to observe changes in DAMGO-induced β-Arrestin 2 recruitment to the MOR, which remains unaffected upon cytokines exposure. However, such lack of effect in β-Arrestin 2 recruitment can be related to assay sensitivity and limited amplification of the signal. In this sense, it is important to notice that the βγ release BRET assay has greater amplification than the β-Arrestin 2 recruitment assay. Therefore, other orthogonal, and more comparable approaches might need to be used to shed light onto this matter. Nonetheless, DAMGO-induced β-Arrestin 2 recruitment is increased in SH-SY5Y cells upon treatment with the cytokines-enriched dialysates derived from DAMGO-treated animals, probably because dialysates might contain other mediators that may influence MOR activity in a manner that β-Arrestin 2 recruitment to the receptor becomes more apparent. These data suggest not only that neuroinflammation-induced changes in opioid tolerance acts through MORs desensitisation, but also that the increase in MOR expression might be a mechanism to counteract this desensitisation, at least, in early opioids exposure. However, the possibility that DAMGO is eliciting the observed effects through a non-MOR-mediated mechanism, while unlikely, cannot be ruled out.

Interestingly, as neuronal MOR activity is tightly related to pain processing [37] and microglia activation-driven neuroinflammation plays a role in the chronicity of pain [38] our results provide mechanistic insight with regards to the relationship between neuroinflammatory processes and MOR anti-nociceptive activity among other related roles of these receptors. Moreover, since microglia might be promoting positive feedback to neurons in a paracrine manner during neuroinflammation in the context of MOR signalling. Indeed, microglial cells and MORs play important roles in reward and thus, in substance use disorders [1], specially under pain conditions [39–40]. Therefore, this mechanistic insight between microglial activation and neuronal MOR activity might be used for future research aiming to find new therapies focusing on analgesia while limiting the development of substance use disorders.

Altogether our and others' data suggest that microglia-neuron communication is bidirectional and might work in a feedback fashion. Through the work presented here we suggest a relationship between microglial activation, cytokine release, and neuronal MOR increased expression and activation. Future research is required to further clarify the nature of this relationship, its temporal dynamics, and its pathophysiological consequences. Moreover, further experiments with opioids used in clinical settings, such as morphine and fentanyl, to induce microglial and MOR activation *in vivo* would also be interesting to elucidate if they replicate the results obtained with the reference agonist DAMGO *in vitro*.

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6. Ethics approval statement

The animal study was reviewed and approved by the Ethics Committee in Experimentation and Animal Welfare of the University of Valencia and the local Government of Valencia (Conselleria d'Agricultura, Desenvolupament Rural, Emergéncia Climática i Transició Ecológica).

CRediT authorship contribution statement

Javier Cuitavi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pere Duart-Abadia:** Methodology, Investigation, Formal analysis. **Julie Sanchez:** Methodology, Investigation, Formal analysis.

Christian M. Sánchez-López: Methodology, Investigation. **Jesús D. Lorente:** Investigation. **Antonio Marcilla:** Resources, Methodology, Funding acquisition. **Isabel Fariñas:** Writing – review & editing, Resources, Methodology. **Meritxell Canals:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Lucía Hipólito:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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