Nanomedicine (Future Medicine)

Title:

MESOPOROUS SILICA PARTICLES ARE PHAGOCYTOSED BY MICROGLIA AND INDUCE A MILD INFLAMMATORY RESPONSE

Running title:

Running title: Microglial response to mesoporous silica particles

Authors:

Júlia Sala-Jarque[1], Elisa García-Lara[1], Paula Carreras-Domínguez[1], Chunfang Zhou[2], Neus Rabaneda-Lombarte[1,3], Carme Solà[3], Jose M Vidal-Taboada[1,4], Adam Feiler[2,5], Ninnie Abrahamsson[6], Elena N Kozlova[6], Josep Saura[1,7 *]

Affiliations:

1 Biochemistry and Molecular Biology Unit, Department of Biomedical Sciences, School of Medicine, University of Barcelona, IDIBAPS, Barcelona, Catalonia, Spain; 2 Nanologica AB, Södertälje, Sweden; 3 Department of Cerebral Ischemia and Neurodegeneration, IIBB, CSIC, IDIBAPS, Barcelona, Catalonia, Spain; 4 Peripheral Nervous System, Neuroscience Department, VHIR, Vall d'Hebron Research Institute, Barcelona, Catalonia, Spain; 5 KTH, Royal Institute of Technology, Stockholm, Sweden; 6 Department of Neuroscience, Uppsala University, Uppsala, Sweden; 7 Institute of Neurosciences, University of Barcelona, Catalonia, Spain.

ORCID IDs:

Carme Solà: 0000-0002-4012-816X

Jose M Vidal-Taboada: 0000-0001-5667-4133

Josep Saura 0000-0002-5601-4466

Elena N Kozlova 0000-0002-2234-0079

(*) Author for correspondence: josepsaura@ub.edu

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Nanologica AB (Södertälje, Sweden) is a company commercializing mesoporous materials for biomedical and life science applications. The authors have no other relevant affiliations or financial involvement with any organization with a financial interest in or financial conflict with the subject matter or materials discussed in the article apart from those disclosed.

Ethical disclosure

"The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Structured abstract (maximum 120 words)

Aim: Mesoporous silica particles (MSPs) are broadly used drug-delivery carriers. In this study, we have analyzed the responses to MSPs of astrocytes and microglia, the two main cellular players in neuroinflammation. Methods: Primary murine cortical mixed glial cultures were treated with Rhodamine-B-labelled MSPs. Results: MSPs are avidly internalized by microglial cells and remain inside the cells for at least 14 days. Despite that, MSPs do not affect glial cell viability or morphology, basal metabolic activity or oxidative stress. MSPs do not affect mRNA levels of key proinflammatory genes but, in combination with LPS, they significantly increase extracellular IL-1β levels. Conclusions: These results suggest that MSPs could be novel tools for specific drug delivery to microglial cells.

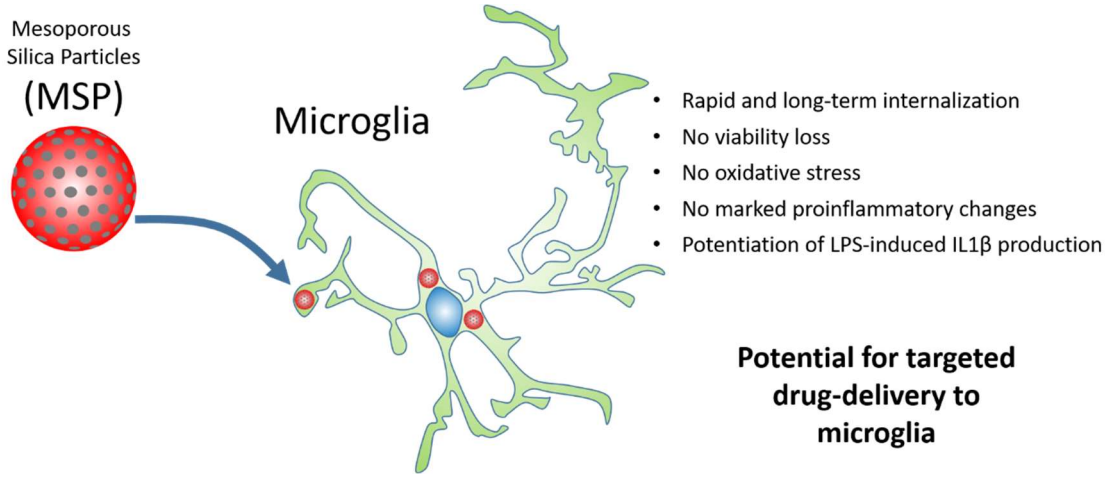
Keywords

Mesoporous silica particles; Microglia, Astrocytes; Neuroinflammation

Lay abstract

Aim: Mesoporous silica particles (MSPs) are broadly used drug-delivery carriers. In this study, we have analyzed the responses of two types of brain cells, astrocytes and microglia, to MSPs. Methods: Mouse astrocytes and microglia were kept alive in culture and were treated with MSPs that were labelled with a red fluorescent agent to facilitate visualization under the microscope. Results: MSPs are avidly internalized by microglial cells and remain inside the cells for at least 14 days. Despite that, MSPs do not affect glial cell viability or morphology, basal metabolic activity or oxidative stress. When given alone, MSPs do not affect mRNA levels of key proinflammatory genes. However, MSPs given in combination with lipopolysaccharide, a strong pro-inflammatory agent, significantly increase the extracellular levels of one of the proinflammatory mediators studied, called IL-1β. Conclusions: These results suggest that MSPs could be novel tools for specific drug delivery to microglial cells.

Graphical abstract



Introduction

Silicon dioxide (SiO_2) or silica can be found in crystalline or amorphous form. Crystalline silica, the major component of sand and rocks, can cause severe pulmonary inflammation [1] and eventually lung cancer [2]. In contrast, amorphous silica is considered to be more inert and safer [3] and it is used in a variety of materials in chemistry, physics, biology and medicine. In recent years, amorphous silica particles of nanometric [4–6] and micrometric [7] size are gaining interest for their potential biomedical applications. A promising application of mesoporous silica particles (MSPs) is as local delivery tools for bioactive peptides. A mesoporous material is defined as a porous material with pore diameters between 2 and 50 nm [8]. MSPs exhibit a series of properties that make them very promising particulate peptide carriers: large surface area, high loading capacity, high possibility of surface modifications, good biocompatibility, tailored pore size and structure, and controlled peptide release [9,10]. We have shown that local delivery in the CNS parenchyma of MSPs loaded with bioactive peptides improves survival of co-implanted motor neuron precursors and increases the life-span of SOD1 mutant mice[11,12]. Therefore, the local and sustained peptide release provided by MSPs could have therapeutic effects in CNS disorders, such as amyotrophic lateral sclerosis (ALS) or spinal cord injury by facilitating local delivery of therapeutic molecules that could not otherwise cross the blood-brain barrier. Also, we have recently demonstrated that implantation of empty MSPs into the spinal cord of a mouse ALS model markedly improves their condition and extends their survival. These findings suggest that empty MSPs may sequester harmful molecules, including pro-inflammatory mediators, thereby attenuating the immune response in the diseased spinal cord [13].

In most CNS disorders, neurons are the cellular target for pharmacological agents. However, when MSPs are locally injected into the CNS parenchyma, they interact with all different kinds of CNS cells. Therefore, it is critical to determine how non-neuronal cells react to MSPs. In this study, we have examined the responses to MSPs of astrocytes and microglia, two glial cell types that are key cellular players in the neuroinflammatory response. Astrocytes are cells of neuroectodermic origin that play key roles in many physiological CNS functions such as ionic homeostasis regulation, energy metabolism and neurotransmission [14]. In response to homeostatic alterations astrocytes react in both neuroprotective and pathological manners [15]. In contrast, the role of microglial cells in normal CNS function is controversial [16]. Although microglia certainly play various physiological roles, such as synaptic pruning and removal of apoptotic cells during neurodevelopment, their main function in the normal CNS is that of a

sensor [17]. Only when they sense alterations in the CNS homeostasis, be it cell damage, cell death, pathogens or abnormal protein deposits, do microglia react, displaying all its biological potential by triggering and orchestrating the neuroinflammatory response [18]. In this response, activated microglial cells upregulate the expression of enzymatic systems with the potential to generate nitrosative and oxidative stress [19], upregulate the expression of proinflammatory mediators [20] and enhance their phagocytic capacity, a defining feature of microglia, to engulf cell debris, pathogens or abnormal bodies [21].

In this study, we have analysed the responses of microglia and astrocytes in primary murine mixed glial cultures to MSPs. Mixed glial cultures are primary cultures mainly composed of astrocytes and microglia. The presence of both cell types is important when modelling neuroinflammation in vitro, since intense cross-talk between astrocytes and microglia occurs in this response [22]. For instance, activated microglia releases IL-1 or IL-18, which, in turn, activate astrocytes [22–25]. Alternatively, activated astrocytes release M-CSF, ATP or C3, which activate microglia [26,27]. As a consequence, the neuroinflammatory response is often potentiated by the presence of both cell types [28] and therefore an in vitro model containing astrocytes and microglia will better reproduce this response. Various studies have analysed the response of microglia to MSPs (see discussion for references) but, to our knowledge, this is the first study using astrocyte/microglia mixed glial cultures in MSP treatments. In summary, MSPs did not cause changes in cell viability, cell morphology or basal metabolism. MSPs were rapidly taken up by glial cells and this internalization was long lasting. Most MSPs were phagocytosed by microglial cells but unequivocal images of MSPs internalized by astrocytes were also observed. Finally, in MSPs-treated cultures we observed a trend for increased proinflammatory cytokine production and oxidative stress generation, and potentiation of lipopolysaccharide (LPS)-induced IL-1β production (Figure 1A).

Methods

Preparation and characterization of MSPs

MSPs were synthesized with the self-assembly templating method [29] following a protocol recently described [13]. Scanning Electron Microscopy was used to characterize the porous surface and particle morphology and this has been described elsewhere[13]. The size of the MSPs was estimated by measuring the diameter from calibrated scanning electron microscopy images using Image J. Rhodamine-B isothiocyanate (Aldrich) was used to label the silica particles. Rhodamine-B (1 mg) was dissolved in methanol (100 mL) and added to 1 g of MSPs-NH2. The mixture was stirred at 40°C for 2 h, filtered, washed with ethanol, and air-dried. The surface area and porosity of Rhodamine-B-labelled MSPs were measured by N2 adsorption-desorption using Micromeritics TriStar II 3020 and they were 402 m^2/g and 21.5 nm, respectively [13]. Rhodamine-B-labelled MSPs were used throughout the study, with the exception of alamar Blue and propidium iodide experiments in which unlabelled MSPs were used because Rhodamine-B fluorescence could have interfered with the readings.

Primary mixed glial cultures

Mixed glial cultures were prepared from postnatal day 1–3 C57BL/6 mice (Charles River) as previously described [30]. Briefly, cerebral cortices were dissected and digested with trypsin followed by repeated pipetting. The resulting single cell suspension was resuspended in fresh culture medium and cultured at 37°C in a 5% CO2 humidified atmosphere. Cells were seeded into 96-well plates (Nunc Multidish, Nunclon Delta) for alamarBlue and propidium iodide experiments, 48-well plates for viability and phagocytosis counts, CellROX, qRT-PCRs, and ELISA experiments, or into polylysine-coated 8-well culture slides (#354118; Corning) for confocal microscopy and time-lapse experiments. The medium was replaced on day in vitro (DIV) 3 and subsequently once a week. Cultures were confluent at DIV 10–12 and were used at DIV21. Astrocytes were the most abundant cell type and microglial cells were 15-20% of total cells.

In vitro treatments

Approximately 5mg of unlabelled or Rhodamine B-labelled MSPs were diluted in 200 µL sterile PBS and MSPs concentration was estimated with a hemocytometer. These stock solutions were further diluted in sterile PBS to prepare working solutions of $1.72x10^7$ MSPs/mL. 1 µL, 5 µL or 10 µL of these working solutions were added per well of a 48 well-plate containing 300 µL of

medium. These treatments are named in this paper 1p, 5p and 10p, respectively. Table 1 shows the corresponding particle concentrations. Rhodamine-B-labelled MSPs were used in most experiments. However, in experiments where Rhodamine-B fluorescence could interfere (propidium iodide staining and alamar Blue), unlabelled MSPs were used. In some experiments, cells were treated with lipopolysaccharide (LPS; *Escherichia coli* 026:B6 serotype; L2654, Sigma-Aldrich) at 100 ng/mL, prepared from a stock solution of 1 mg/mL sterile medium. Stock solutions of MSPs or LPS were kept at -20°C.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeated by incubation with 100% methanol for 7 min. The cells were then incubated overnight at 4°C with primary antibody: rabbit anti-GFAP (Glial Fibrillary Acidic Protein; 1:1000, DakoCytomation); rabbit anti-Iba1 (1:500, Wako); rat anti CD68 (1:1000, Serotec). Cultured cells were then incubated for 1h at room temperature with Alexa Fluor 488-labeled goat anti-rabbit or anti-rat secondary antibody (1:1000, Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI, 1:2000, Sigma-Aldrich). All antibodies were diluted in 0.01 M phosphate buffered saline containing 7% normal goat serum and 0.01% thimerosal (Sigma-Aldrich). Immunocytochemistry was visualized with an inverted fluorescence microscope Olympus IX70 (Olympus) coupled to a digital camera CC-12 (Olympus Soft Imaging Solutions GmbH) or with a multiphoton and high-velocity spectral confocal microscope (Leica TCS-SP5; Advanced Optical Microscopy Facility, CCiT-UB).

Propidium iodide staining

Cells were seeded in 96-well culture plates and treated with unlabelled MSPs for 24 h. As a negative control, cells were treated with culture medium and as a positive control of cell death cells were treated with 1% Triton-X-100. Cells were then incubated with Propidium iodide (7.5 µg/mL, Molecular Probes) and Hoechst 33342 (3 µg/mL; Molecular Probes) for 10 minutes to label the nuclei of dead and total cells, respectively.

Cell and MSPs counts

In all experiments, 3-4 wells per condition were used and 3-4 randomly selected fields of 145.000 μm^2 were photographed per well. Images were acquired with an inverted fluorescence

microscope Olympus IX70 (Olympus) coupled to a digital camera CC-12 (Olympus Soft Imaging Solutions GmbH). Image analysis was carried out with ImageJ software (version 1.50g) using the "Cell counter" plugin. Counting was made manually by an experimenter blind to the treatments. The following parameters were quantified: total number of cells (by DAPI staining); the number of microglial cell (by Iba1 staining); the number of cells with MSPs (by Rhodamine-B signal); distinguishing microglial from non-microglial cells (by Iba1 staining); the number of cells showing oxidative stress (by CellROX Green staining); the number of particles internalized per cell (by counting Rhodamine-B positive particles per cell), and finally distinguishing microglial from non-microglial cells (by Iba1 staining) along with oxidative stressed and non-stressed cells (by CellROX Green staining).

Time-lapse Laser Scanning Confocal Microscopy

Mixed glial cultures were grown, as described above, onto polylysine-coated 8-well culture slides (#354118; Corning). Automated multiposition live cell imaging was carried out using a Leica TCS SP5 confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with Adaptive Focus Control to keep the specimen in focus. Rhodamine-B images were acquired sequentially line by line using laser line and detection range at 570-650 nm. Multiposition confocal images were acquired every 2 min during 12-14 h at 512x512 pixel format and 400Hz using a 63x oil immersion objective lens (NA 1.3) and pinhole set at 2 Airy units. Bright field images were acquired simultaneously. All experiments were performed at 37^{o}C in a humidified atmosphere of 5% CO_2.

Alamar Blue assay

Alamar Blue^{TM} (ThermoFisher Scientific) is a redox indicator used to measure metabolic activity and cell viability. Its active compound resazurin is stable in culture medium, non-toxic to the cells, permeable to the cell membrane and weakly fluorescent. Due to the cellular activity, resazurin is reduced to the highly fluorescent resorufin. Mixed glial cultures were seeded in 96 well-plates and treated with vehicle or MSPs for 24h. Ten µL of alamar Blue reagent were added per well 4 h prior to reading at 560nm excitation and 590nm emission wavelengths (Multiskan Spectrum, Thermo Electron Corporation). As a positive control of cell death 10µL Triton-X-100 20% were added per well 30 min before the addition of alamar Blue reagent. Negative controls included wells containing culture medium and no cells, with or without MSPs.

CellROX Green staining

Oxidative stressed cells were identified using the reactive oxygen species (ROS) indicator CellROX® Green reagent, following the manufacturer's recommendations (ThermoFisher Scientific). After treatments, cells were incubated with 5μM CellROX® Green reagent for 30 min in the cell culture incubator and fixed for 20min with 4% paraformaldehyde. Nuclei were then stained with DAPI 1:5000 for 20 min to quantify the number of total cells and the percentage of CellROX positive cells. Cultures stained with CellROX Green reagent and DAPI were photographed not later than 72h after fixation. In preliminary experiments, we had observed that the level of CellROX Green fluorescence intensity in fixed cells increased upon illumination for 20-30 secs and then plateaued. Photographs were therefore taken after 60 secs of constant illumination in randomly selected fields not previously visualized under the fluorescence microscope.

qRT-PCR

The mRNA expression of pro-inflammatory markers was assessed by quantitative real-time PCR. RNA was isolated from 6 wells per condition (48 well-plates) using High Pure RNA Isolation Kit (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland). RNA (0.5-0.75 μg) was reverse transcribed with random primers using Transcriptor Reverse Transcriptase (Roche Diagnostics). cDNAs (2-3 ng) were used to perform qRT-PCR as previously described [31]. Table 1 shows the primers used in this study (Integrated DNA Technology, IDT, Skokie, IL, USA). β-Actin and Rn18s were used as reference genes and the $2^{-\Delta\Delta Ct}$ method was used to estimate relative gene expression values [32].

ELISA

The concentration of the proinflammatory cytokines TNFα, IL-6 and IL-1β in the culture medium was analyzed by sandwich ELISA kits according to the manufacturer instructions (Murine TNF-α Mini TMB ELISA Development Kit, 900-TM54, Peprotech; Murine IL-6 Mini TMB ELISA Development Kit, 900-TM50, Peprotech; Mouse IL-1β / IL-1F2 DuoSet ELISA, DY401.05, R & D Systems). Media conditioned by mixed glial cultures treated for 24h with vehicle, MSPs, LPS, or LPS+MSPs were used. To keep the absorbances of the samples within the range of the standard

curve, conditioned medium was diluted in cultured medium 1/5, 1/3 or left undiluted for the determination of TNFα, IL-6 or IL-1β, respectively.

Statistics

Statistical analyses were performed using one-way or two-way analyses of variance (ANOVA) followed by Bonferroni post-test using GraphPad Prism software. Values of $p<0.05$ were considered statistically significant. Results are presented as the mean + SEM.

Results

MSPs synthesis and properties

MSPs were synthesized as indicated in Methods. In previous studies from our group the properties of these particles have been described in detail [13]. The average size of the MSPs was 4.1 ± 1.2 µm (SD, n=252, range 1.9-7.7µm). The Brunauer–Emmett–Teller surface area was 402 m^2/g and the pore size was 21.5 nm. Representative scanning electron microscope images of the MSPs used in this study are shown in figure 1B, C.

MSPs do not affect viability and morphology in cultured glial cells.

We were first interested in establishing the effects of MSPs on cell viability of glial cells in vitro. To this end primary mixed glial cultures were treated for 2, 7 or 14 days with two concentrations of MSPs named 1p and 10p (see table 1 for units). The total cell number, assessed by counting DAPI positive nuclei, was not affected by MSPs at any of the times and concentrations tested (Figure 2A). Mixed glial cultures are composed mainly of astrocytes and 15-25% microglial cells. Astrocytes are easy to label by GFAP, but almost impossible to count due to high density and overlapping profiles whereas microglial cells are smaller and less abundant and can be counted reliably after immunostaining. To discard a specific effect of MSPs on microglia viability, which could be masked by the larger astroglial numbers when counting total cells, we counted the number of microglial cells identified by Iba1 immunostaining. As seen in figure 2B the proportion of microglial cells was also not affected by MSPs at any of the times and concentrations tested. These findings suggest a lack of cytotoxic effects of MSPs on mixed glial cultures. This result was confirmed by propidium iodide staining 24 h after MSPs treatment which showed virtually no propidium iodide positive cells in cultures treated with MSPs at 1p or 10p, similarly to vehicle-treated cultures (Figure 2C). Finally, immunocytochemistry staining with anti-Iba1 or -GFAP antibodies **revealed no overt MSPs-induced morphological changes in microglial cells (Figure 2D) or astrocytes (not shown), respectively**.

MSPs are taken up by microglia in primary mixed glial cultures

An immediate observation from these experiments was that MSPs, which were easily visualized by their Rhodamine-B labeling, were taken up by cultured cells (see time-lapse video in Supplementary File 1). By combining immunocytochemistry for the microglial marker Iba1 with

Rhodamine-B visualization we estimated the number of microglial and non-microglial cells that had internalized MSPs, and the average number of MSPs per cell in mixed glial cultures treated with MSPs (1p or 10p) for 2, 7 and 14 days. As expected, the number of microglial cells with particles was significantly higher ($p<0,001$ for the concentration factor in the two-way ANOVA) in cultures treated with the high MSPs concentration 10p (49.2% cells, average for the 3 time points) than with the low 1p concentration (7.8%, average for the 3 time points) (Figure 3A). Somewhat unexpectedly, the proportion of MSP-positive microglial cells was not significantly different when comparing cultures treated with MSPs for 2, 7 or 14 days (Figure 3A). We then counted the number of MSPs per cell. In cultures treated with 10p the average number of MSPs per microglial cell was 3.0, 2.3 and 2.2 particles/cell at 2, 7 or 14 days, respectively, with no significant differences between treatment times (Figure 3B, C) suggesting that once particles are taken up by microglia they remain stably inside the cell for many days. This analysis also revealed **the presence of MSPs in cellular profiles with no Iba1 immunoreactivity, therefore in non-microglial cells.** MSPs in non-microglial cells were much less common than in microglia and the average number of MSPs per non-microglial cell was 0.07, 0.12 and 0.08 particles/cell for the 3 times analyzed (Figure 3B).

Internalization kinetics of MSPs at early time points

Based on these data we selected for future experiments an MSPs concentration half the maximum concentration used in the previous experiments (10p). This MSPs concentration, named hereinafter 5p, corresponds to 285000 MSPs/mL and guarantees a lack of effect on viability and a marked internalization by mixed glial cultures. Since in the first time-course experiment we observed a similar level of MSPs internalization in the three time-points studied (2, 7 and 14 days) we were interested in the study of the kinetics of internalization at times shorter than 48 h. In this case, we observed a clear time-dependent MSPs internalization with a plateau around 24 h both in the number of microglial cells with MSPs (Figure 4A) and in the number of MSPs per microglial cell (Figure 4B). The comparison of the number of MSPs per cell revealed again a **much higher number of MSPs in microglia than in non-microglial cells.** In contrast to the kinetics of internalization in microglia, the maximum number of MSPs in non-microglial cells was achieved at the earlier time-point and declined thereinafter (Figure 4B). This suggests that many particles seen in non-microglia at early time-points are simply attached to the astroglial surface but not internalized. The fact that their numbers decline with time reflects internalization by neighbouring, actively moving microglial cells. This is also suggested by time-lapse images of mixed glial cultures treated with MSPs (Supplementary File 1)

In order to demonstrate that MSPs were indeed internalized by cells, we performed confocal microscopy on mixed glial cultures treated for 24 h with Rhodamine-B-labelled MSPs (5p) and immunostained with Iba1 or GFAP to identify microglia and astrocytes, respectively. As expected, Iba1 images unequivocally showed the presence of MSPs inside microglial cells, often clustered in a perinuclear location or in distal terminals (Figure 4C). GFAP images showed that most MSPs were not associated with astrocytes. However, clear examples of MSPs inside astrocytes were observed (Figure 4D), demonstrating that **astrocytes, although not professional phagocytes, are able to internalize MSPs.**

Effects of MSPs on basal metabolic activity and oxidative stress

Internalization of MSPs by glial cells could cause cellular stress resulting in metabolic alterations and oxidative stress. To test the potential effects of MSPs on metabolic function we performed the alamarBlue assay [33]. This method is often used to estimate cell viability and cytotoxicity. Since we had already determined by cell counts that cell viability was not affected by MSPs in our model, changes in alamarBlue signal would reflect changes in basal metabolic activity. We did not observe differences in alamarBlue signal in mixed glial cultures treated for 24h with vehicle or MSPs 5p (10988 ± 331 vs 11543 ± 546, n=4, (SD)). Similar results were obtained with the MTT assay (data not shown). These data indicate that **MSPs do not have significant effects on basal metabolic activity in mixed glial cultures.**

We next used the CellROX Green assay to analyse whether MSPs induce oxidative stress in glial cells. In this experiment, and in all subsequent experiments, apart from control and MSPs conditions, treatments with LPS and LPS+MSPs were also included. The aim of these two new conditions was to evaluate whether MSPs had any potentiating or attenuating effects on the well-known activating effects of LPS on glial cells. As shown in figure 5A, cells with oxidative stress were clearly identified by a strong CellROX positive fluorescent signal and represented a small fraction of total cells. The number of CellROX positive cells did not significantly differ between control and MSPs conditions, LPS increased the number of CellROX positive cells and no differences were observed between LPS and LPS + MSPs (Figure 5A,B). We then assessed the CellROX status (positive or negative) and the number of MSPs/cell within the population of cells that had internalized MSPs. In cultures treated with MSPs (5p) the percentage of CellROX-positive and -negative cells in the population of cells with internalized MSPs was 35.7 ± 13.7% and 64.3 ± 13.7%, respectively. In LPS+MSPs-treated cultures these proportions were 46.6 ±

15.3% and 53.4 ± 15.3%. The results of the proportion of MSPs-positive cells grouped by CellROX status and number of MSPs are shown in figures 5C and 5D. In cultures treated with MSPs only (Figure 5C) 44.3% of cells had 2-5 MSPs, 26.2% of cells had 1 MSPs/cell and 29.5% had >5MSPs/cell. The proportions of CellROX-positive and -negative cells were very similar in the three classes indicating that a higher number of MSPs/cell does not result in a higher proportion of CellROX positive cells. Similar findings were obtained when analysing cells treated with LPS+MSPs (Figure 5D). In this condition, the proportion of CellROX-positive cells with high number of MSPs was higher than in the MSPs-only condition. However, there was not a significant effect on the number of MSPs internalized per cell in CellROX status as seen in the MSPs-only treatment. Altogether, these results suggest that **MSPs do not cause oxidative stress in glial cells.**

Effects of MSPs on proinflammatory gene expression in mixed glial cultures

Finally, we were interested in studying whether MSPs are able to induce, per se or in combination with LPS, a proinflammatory reaction in mixed glial cultures. We first analysed by qRT-PCR the mRNA levels of the proinflammatory genes TNFα, IL-6, IL-1β and COX2 in mixed glial cultures treated for 6h with vehicle, MSPs (5p), LPS or LPS+MSPs. MSPs alone did not induce significant changes in the levels of these mRNAs, although for all the genes analysed the mean mRNA levels were higher in cultures treated with MSPs than in vehicle-treated cultures (Figure 6). In combination with LPS, MSPs did not significantly potentiate or attenuate the LPS-induced upregulation of the mRNAs of the proinflammatory cytokines TNFα, IL-1β or IL-6. In the case of COX2, LPS did not cause a significant increase in the mRNA levels whereas LPS+MSPs did, but the difference between LPS and LPS+MSPs was not statistically significant (Figure 6).

We then analysed by ELISA the protein levels of the proinflammatory cytokines TNFα, IL-6 and IL-1β in the conditioned media of mixed glial cultures treated for 24 h with vehicle, MSPs (5p), LPS or LPS+MSPs. **MSPs alone did not induce any change in cytokine levels. In combination with LPS, MSPs did not affect the levels of TNFα and IL-6 but it significantly increased the levels of IL-1β in the conditioned media** (Figure 7).

Discussion

MSPs hold great potential for the long-lasting delivery of drugs, including bioactive peptides. This approach has been successfully used in various experimental in vivo models to deliver anti-microbial peptides [34], anti-inflammatory peptides [35] or insulin [36]. In the CNS, local injection of peptide-loaded MSPs has the additional advantage of allowing the delivery to the site of lesion of bioactive peptides that could not otherwise cross the blood-brain barrier. Using this approach, injection into the CNS parenchyma of MSPs loaded with neurotrophic peptide mimetics has enabled functional differentiation of motor neurons from transplanted embryonic stem cells [11] and supported sensory axon regeneration [12]. Interestingly, empty MSPs have also been observed to induce positive effects on neuronal survival [13,37]. When considering the local delivery of MSPs into the CNS parenchyma as a therapeutic approach, it is important to ascertain how the various CNS cell types react to these particles. The present study is focused on the responses of microglia and astrocytes to MSPs.

Several studies have analysed the responses of microglia to silica particles using rat or mouse primary microglial cultures [35,38–40], the N9 microglial cell line [41,42] or organotypic cultures [43]. The silica particles used in these studies ranged between 20 nm and 200 nm in diameter, which is smaller than the 3-10 µm diameter of the particles used in our study. In these studies MSPs were consistently internalized by microglial cells [38,40–43]. In contrast, analyses of viability and proinflammatory and oxidative responses induced by MSPs in microglia showed less consistent findings. MSPs did not affect microglial viability in two studies[35,38] but induced pyroptosis in another [42]. MSPs induced microglial expression of the proinflammatory cytokines TNFα, IL-1β and IL-6 in some studies [38,39,42] but not in others [35,41,43]. Similarly, MSPs-induced ROS and RNS production by glial cells has been reported in some studies [38,42] but not in others [39].

Our findings are in keeping with the reported findings on the effects of silica particles on microglial cells, namely avid internalization, lack of effects on cell viability and mild, if any, effects on proinflammatory cytokine and ROS/RNS production. It is important to remember that in our study these effects were analysed for the first time with large particles (3-10 µm diameter) and with mixed glial cultures that contain not only microglia, but also astrocytes. As reported in the abovementioned studies, we did not observe any detrimental effect of MSPs on glial cell viability. Even though MSPs remained internalized by microglial cells for up to 14 days the

particles did not affect cell viability or morphology. This is a positive finding when considering the use of these particles as delivery agents of bioactive peptides since it suggests that microglia and astrocytes tolerate well the prolonged presence of these particles.

We have also observed that MSPs were avidly internalized by microglial cells, with most MSPs being internalized after 24h of exposure. This observation agrees with the high phagocytic capacity of microglia and with all previous literature describing the interaction of microglial cells with silica particles [38,40,41,43]. If confirmed in vivo, microglial internalization of MSPs needs to be considered for future in vivo studies. On the one hand, the functional results obtained in vivo with MSPs loaded with neurotrophic peptides [11,12] indicate that the bioactive peptides were released from the MSPs in sufficient concentration and time to exert their effects on target cells, such as neurons or stem cells. On the other hand, considering that MSPs allow the sustained release of the loaded molecules for periods of several days or even months [44–46], microglial internalization of MSPs is certainly decreasing the efficacy of such treatments. Minimization of microglial MSPs internalization would probably boost the therapeutic properties of peptide-loaded MSPs in vivo. Strategies to reduce microglial internalization of MSPs include co-loading MSPs with drugs inhibiting microglial internalization of the particles or chemical modifications of the particles such as polyvinylpyrrolidone coating [40]. Microglial internalization of MSPs is certainly a drawback for the use of MSPs as drug delivery tools in the CNS. However, this could also be seen as an opportunity since MSPs could be loaded with peptides or other therapeutic molecules targeting microglial cells. To the best of our knowledge, little has been done regarding the investigation of such therapeutic potential. Nevertheless, targeting microglial cells could be used to deliver to microglia molecules that do not cross well the plasma membrane with the advantage that this delivery would be cell-specific since non-microglial CNS cells would poorly internalize the particles. For example, it is known that in neurodegenerative diseases, such as ALS, the production of oxidant species by microglia increases, which can be damaging to brain cells. In a previous study [47], silica particles were used to deliver an antioxidant compound that reduced the pro-inflammatory activation of the microglial cells, which were previously stimulated by the authors. Moreover, MSPs labelled with gadolinium have been used to track in vivo microglial accumulation in glioma tumours in mice [48], and PLGA nanoparticles have been successfully used to deliver small-interfering RNA to microglia [49,50]. Our observation that MSPs remain inside microglial cells for at least 14 days, along with the fact that they do not show obvious harmful effects, suggests a long-lasting action on microglia by the drugs loaded into the MSPs. To what extent these in vitro findings of

microglial behaviour reflect the in vivo situation remains to be clarified. Studies are needed to elucidate the best sort of particles for microglial targeting, in terms of composition, size or pre-treatment, as well as issues such as cargo and particle degradability by microglial cells.

The possibility that MSPs could induce oxidative or nitrosative stress in glial cells is of importance. Microglia has a high ability to generate reactive oxygen and nitrogen species (ROS and RNS) [51], major effectors of the detrimental consequences of the neuroinflammatory response [52]. To our knowledge, two studies have analyzed the effects of silica particles on glial ROS and RNS generation in primary rodent microglial cultures. Choi et al. [38] reported increased ROS and RNS production whereas Xue et al. [39] reported a lack of NO generation. In our study, MSPs did not induce NO production in mixed glial cultures nor did they cause a significant increase in the number of cells with oxidative stress. These observations suggest that the large MSPs used in this study do not induce significant nitrosative or oxidative stress in glial cells, which is a positive finding when considering the in vivo use of MSPs in the CNS. It is important to note that these experiments were performed after short-term MSPs exposures. Further studies with long-term exposures are required to discard glial ROS or RNS generation induced by chronic exposure to MSPs.

Production of ROS and RNS by glial cells is probably the main neurotoxic element within the neuroinflammatory response. A sensitive indicator of the existence of this response is the increased expression of genes encoding major proinflammatory mediators, which can occur prior or even in the absence of ROS and RNS production. Our findings indicate that MSPs do not induce a robust neuroinflammatory response in mixed glial cultures. We did not observe significant MSPs-induced upregulation of the proinflammatory cytokines TNFα, IL-1β and IL-6, or of the PGE2-producing enzyme COX-2, which are sensitive markers widely used to reveal the inflammatory character of a biological response in the CNS [53]. Although minor increases in TNFα, IL-1β and IL-6 have been reported in culture microglia treated with silica particles [38,39], most studies have reported the absence of an MSPs-induced proinflammatory response in microglia [35,41,43]. A lack of activation of the neuroinflammatory program by MSPs is in principle a positive observation when considering the use of MSPs as drug delivery tools or sequestering devices in the CNS.

The only significant response induced by MSPs with a pro-inflammatory character that we have observed is the increase in the concentration of extracellular IL-1β in mixed glial cultures treated

with LPS. This increase in IL-1β protein levels was not observed at the mRNA level, suggesting a post-transcriptional effect, and it was observed for IL-1β but not for the other pro-inflammatory cytokines analysed, namely IL-6 and TNFα. Both the post-transcriptional character and the IL-1β specificity suggest that the effects of MSPs on IL-1β protein levels are mediated by activation of the inflammasome, a large multiprotein complex that cleaves pro-IL-1β precursor into biologically active IL-1β and it does not mediate IL-6 or TNFα activation [54]. This is supported by the fact that silica particles are activators of NLRP3 inflammasomes [55] and by a previous report showing IL-1β secretion induced by nano-silica in human THP1 macrophages primed with LPS in the absence of increased IL-1β mRNA levels [56]. The increased production of IL-1β in activated microglia induced by MSPs is a potentially deleterious effect, since IL-1β is a master activator of the neuroinflammatory response [57]. In agreement with this, IL-1β blockade or deficiency is protective in animal models of amyotrophic lateral sclerosis [58] and multiple sclerosis [59]. However, the situation is not that simple and IL-1β has been shown to promote remyelination in the adult CNS [60], to elicit a neuroprotective effect in a Parkinson's disease model [25] and to favour a trophic phenotype of cultured human mesenchymal stem cells for CNS therapy [61]. The overall effect of IL-1β in a given situation is probably dependent on the local concentrations of IL-1β achieved as well as on cell-specific effects. If MSPs are injected into the CNS it is important to determine IL-1β extracellular levels and, if increased, to consider its potential effects.

Conclusions and Future Perspective

In conclusion, this study shows that the interaction of MSPs with astrocytes and microglia in culture does not induce marked changes in viability, morphology, oxidative stress and pro-inflammatory gene expression in these cells, with the exception of a potentiation of LPS-induced IL-1β production. A marked internalization of MSPs is observed in microglia, and to a much lesser extent, in astrocytes. The use of silica particles as drug-delivery carriers holds great potential and it will certainly be the subject of future studies. When considering the CNS as the target tissue, strategies to reduce glial internalization of these particles could be particularly useful to enhance their potential for drug delivery to neurons. On the other hand, the results from our study open the possibility that MSPs could be used for targeted drug delivery to microglial cells.

Summary points

- Mesoporous silica particles (MSPs) are broadly used drug-delivery carriers with large surface area and variable pore size.
- When applied into the CNS it is important to ascertain the responses of neuronal and non-neuronal cells to MSPs.
- In this study, we have analyzed the responses to MSPs of astrocytes and microglia, the two main cellular players in the neuroinflammatory response.
- In primary murine cortical mixed glial cultures MSPs did not affect glial cell viability or morphology.
- MSPs were avidly taken up by microglial cells and to a much lesser extent by astrocytes.
- MSPs did not affect basal metabolic activity nor did they induce marked oxidative stress.
- MSPs did not affect mRNA levels of the proinflammatory genes TNFα, IL-6, IL-1β and COX2 nor did they potentiate the LPS-induced upregulation of these mRNAs, although some not significant trends were observed.
- MSPs did not induce changes in TNFα, IL-1β or IL-6 protein levels in the conditioned media, but, in combination with LPS, MSPs significantly increased IL-1β levels in the conditioned media.
- These results suggest that MSPs could be novel tools for specific delivery of peptides or other agents to microglial cells.

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In a rat spinal nerve ligation in vivo model, miRNA-loaded silica nanoparticles injected intrathecally into the spinal cord attenuate microglia inflammatory response

Table 1. Concentrations of MSPs used in this study

Code	Particles/volume (MSPs/mL)	Particles/area (MSPs/cm^2)	Mass/volume (μg/mL)
1p	57000	17100	0.77
5p	285000	85500	3.88
10p	570000	171000	7.76

Figures

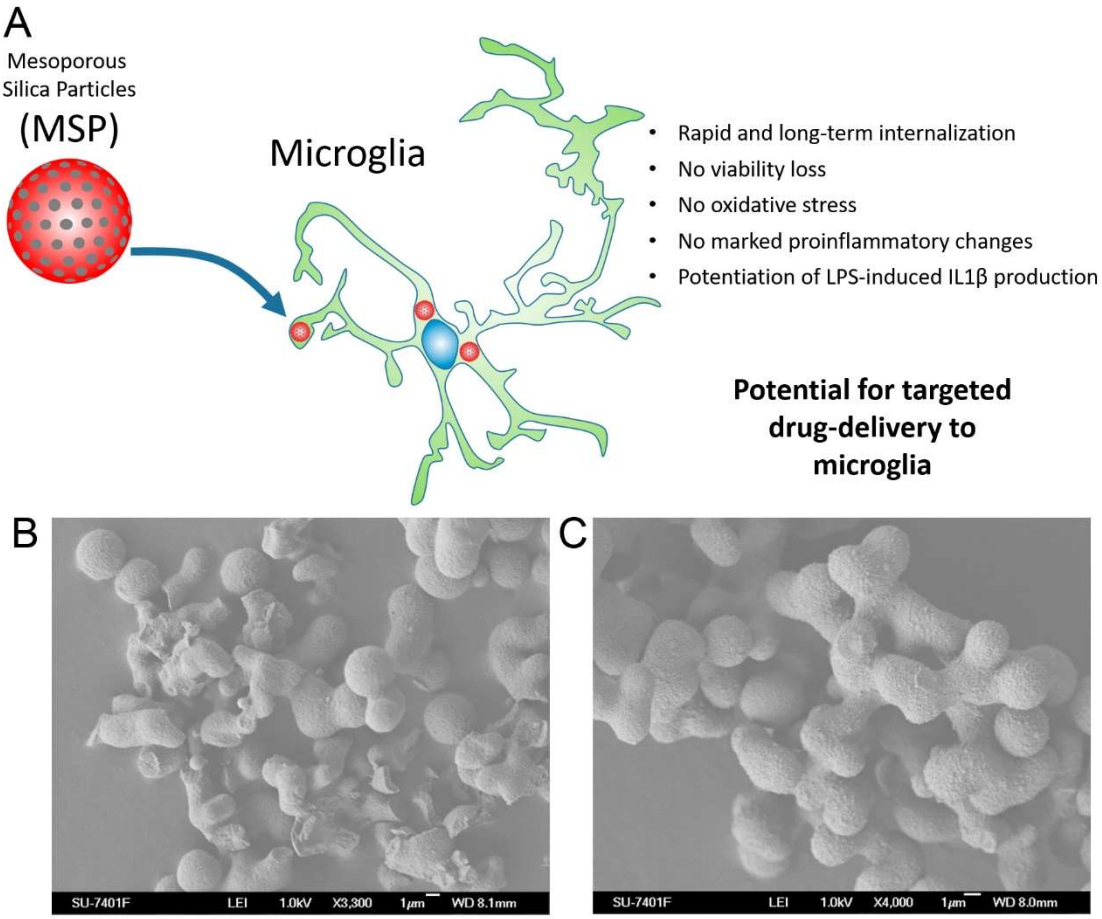

Figure 1. A) The main findings of this study are graphically summarized in this figure. B, C) Representative scanning electron microscope images of the MSPs used in this study.

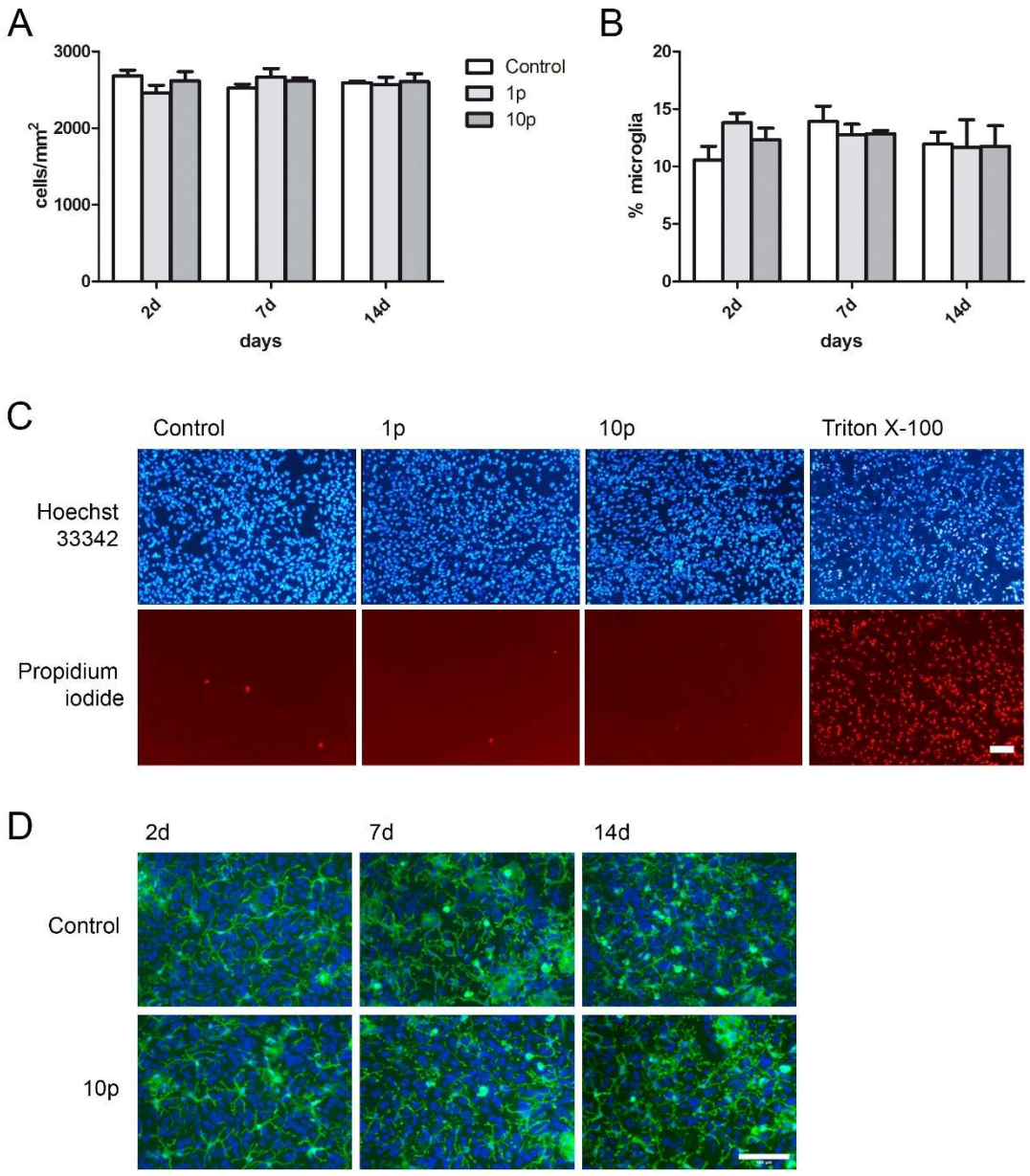

Figure 2. Absence of effects of MSPs on glial cell viability and morphology. A) Primary mixed glial cultures were treated for 2, 7 or 14 days with vehicle (control) or MSPs (1p and 10p). Total cell counts, estimated by DAPI staining, are shown as mean + SEM, n=3 independent cultures. B) Proportion of microglial cells in mixed glial cultures, treated as in A, estimated by counting Iba1-positive cells vs total cells. Data is shown as mean + SEM, n=3. No statistically significant differences were found in the analysis of data shown in A or B by two-way ANOVA. C) Representative images of mixed glial cultures treated for 24h with vehicle or MSPs and stained with Hoechst-33342 to show all cell nuclei and with propidium iodide to show nuclei of non-viable cells. Triton X-100 treatment is used as positive control of cell death. In vehicle-treated cultures cell death is minimal and is not increased by treatment with MSPs. Magnification bar,

100 µm. D) Representative images of mixed glial cultures treated for 2, 7 or 14 days with vehicle (control) or MSPs (10p) and immunostained with Iba1 to show microglial cells (green). Total nuclei were label with DAPI (blue). Treatment with particles does not induce overt changes in microglial morphology. Magnification bar, 100 µm.

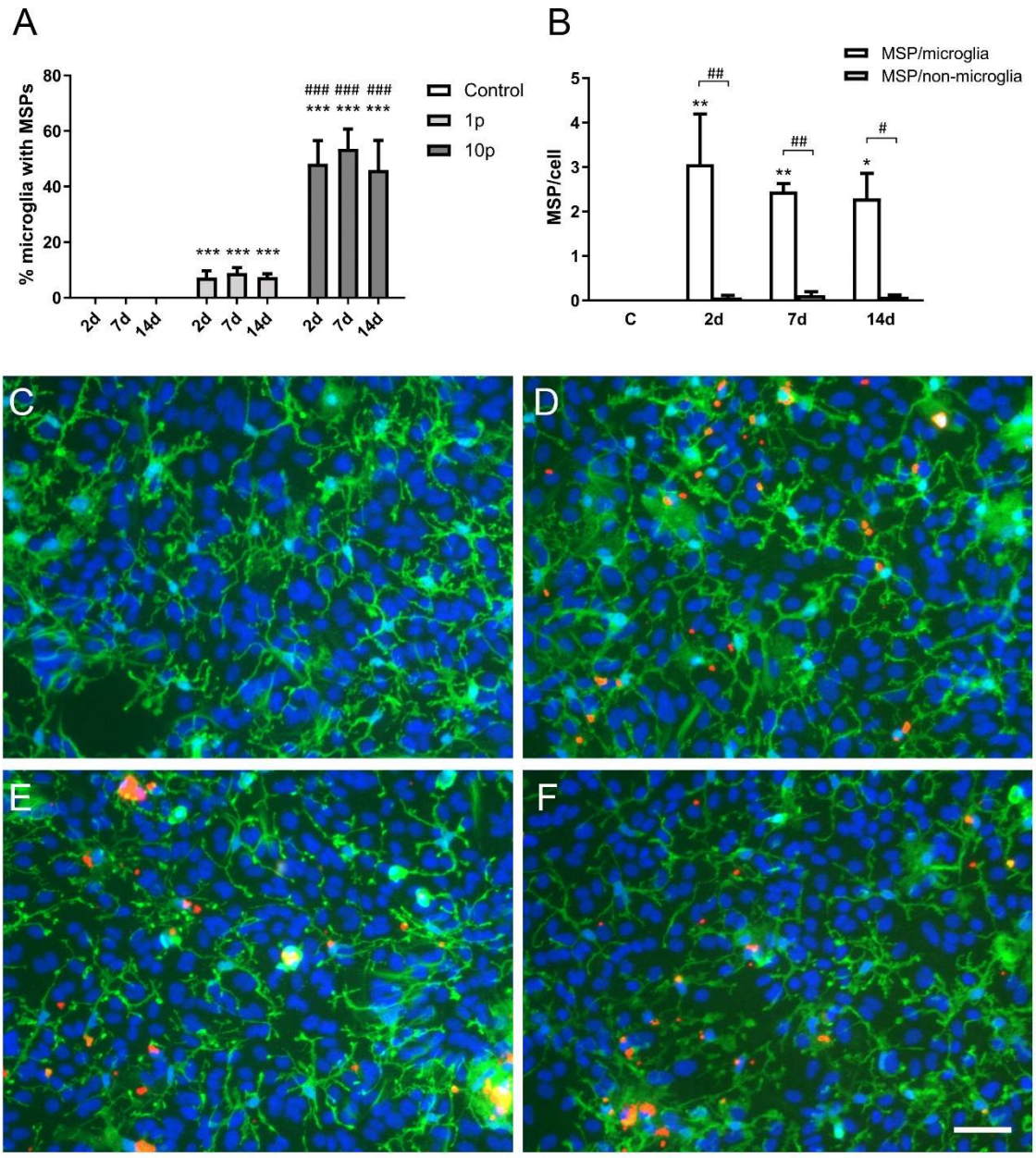

Figure 3. Internalization of MSPs by mixed glial cultures: long-term time-course. Primary mixed glial cultures were treated for 2, 7 or 14 days with vehicle (control) or MSPs (1p and 10p). A) Percentage of microglial cells, identified by Iba1 immunostaining, containing particles. Treatment with MSPs at 10p concentration results in more microglial cells with particles than 1p treatment, but the proportion of microglial cells with particles does not change significantly over time. Data are shown as mean + SEM, n=3 independent cultures. *** p<0.001 vs control; ### p<0.001 vs respective 1p condition. Two-way ANOVA. B) Number of MSPs per microglial and non-microglial cell. The average number of particles is much higher in microglia than in non-microglial cells and this number does not change significantly over time. Data are shown as mean + SEM, n=3 independent cultures. * p<0.05, ** p<0.01 vs control; # p<0.05, ## p<0.01 vs

MSPs/non-microglial cells in the respective condition. Two-way ANOVA. C-F) Representative images of mixed glial cultures treated with vehicle (C) or MSPs at 10p concentration for 2 days (D), 7 days (E) or 14 days (F). Microglia are immunostained for Iba1 (green), nuclei are shown with DAPI (blue) and MSPs are visualized by Rhodamine-B labelling (red). Magnification bar, 100 µm.

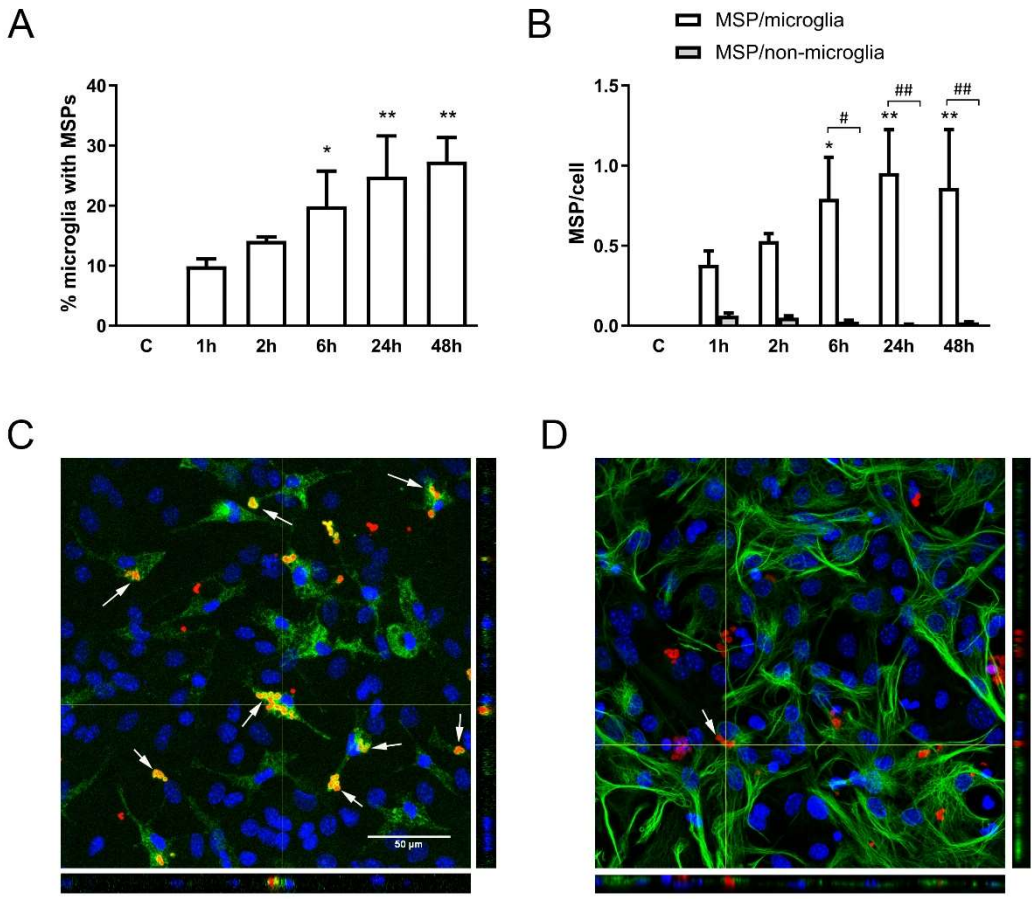

Figure 4. Internalization of MSPs by mixed glial cultures: short-term time-course. Primary mixed glial cultures were treated for 1-48 h with vehicle (control) or MSPs (5p). A) Percentage of microglial cells, identified by Iba1 immunostaining, containing particles. A time-dependent increase in the number of microglial cells with particles is observed. Data are shown as mean + SEM, n=3 independent cultures. * $p>0.05$, ** $p<0.01$ vs control. One-way ANOVA. B) Number of MSPs per microglial and non-microglial cell. The average number of particles is much higher in microglia than in non-microglial cells. In microglial cells, but not in non-microglial cells, this number increases in a time-dependent manner. Data are shown as mean + SEM, n=3 independent cultures. * $p<0.05$, ** $p<0.01$ vs control; # $p<0.05$, ## $p<0.01$ vs MSPs/non-microglial cells in the respective condition. One-way ANOVA. C) Confocal image of a mixed glial culture treated for 24 h with MSPs (5p). Microglia are immunostained for CD68 (green), nuclei are shown with DAPI (blue) and MSPs are visualized by Rhodamine-B labelling (red). The field shows various examples of microglial cells with internalized particles (arrows). Magnification bar is valid also for D, 50 µm. D) Confocal image of a mixed glial culture treated for 24 h with MSPs (5p). Astrocytes are immunostained for GFAP (green), nuclei are shown with DAPI (blue) and MSPs are visualized by Rhodamine-B labelling (red). Most MSPs are not associated with

astroglial profiles, but some are. Arrow points to a group of 5-6 particles clearly inside an astroglial cell as shown by their perinuclear location and by the XZ and XY projections.

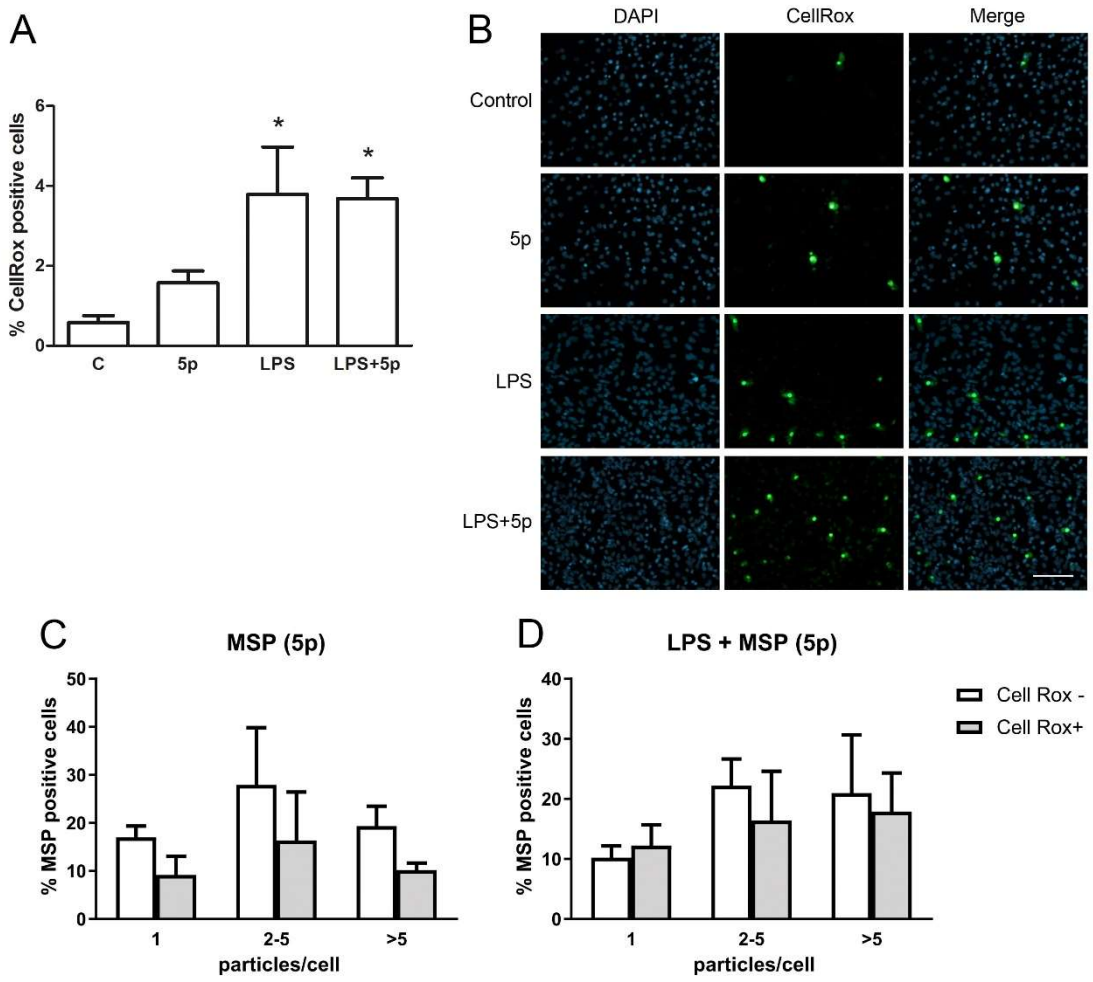

Figure 5. Oxidative stress in mixed glial cultures treated with MSPs. A) Percentages of CellRox positive cells in mixed glial cultures treated for 24 h with vehicle (C), MSPs (5p), 100 ng/mL LPS (LPS) or LPS + MSPs (LPS+5p). Data are shown as mean + SEM, n=7 independent cultures. * $p<0.05$ vs vehicle. One-way ANOVA. B) Representative images of mixed glial cultures treated for 24 h as described in figure 4A and stained with DAPI (blue) to visualize total cell nuclei and CellRox (green) to reveal cells with oxidative stress. Magnification bar 100 µm. C,D) Mixed glial cultures were treated for 24 h with MSPs 5p (C) or LPS + MSPs 5p (D). The graphs show the percentages of cells, within the population of cells with particles, according to their number of particles and CellRox staining. Data show mean + SEM, n=3 independent cultures.

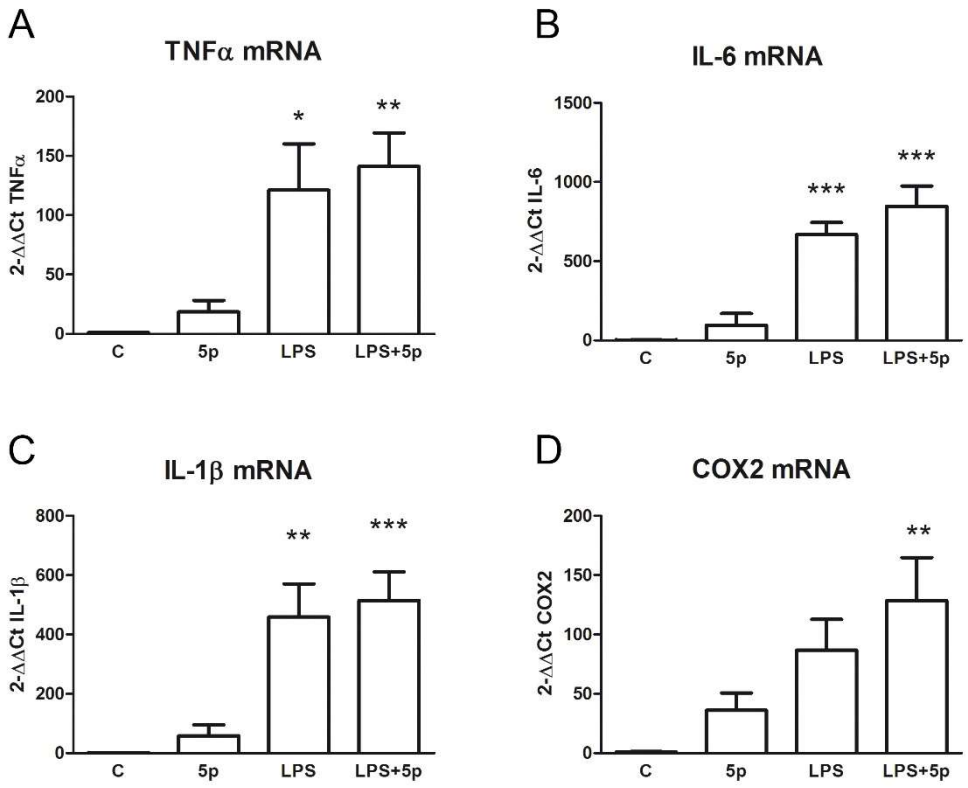

Figure 6. Effects of MSPs on glial proinflammatory genes mRNA levels. Mixed glial cultures treated for 6 h with vehicle (C), MSPs (5p), 100 ng/mL LPS (LPS) or LPS + MSPs (LPS+5p). mRNA levels of the proinflammatory genes TNFα (A), IL-6 (B), IL-1β (C) and COX-2 (D) were analyzed by qRT-PCR. Data are shown as mean + SEM, n=5-6 independent cultures. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control. One-way ANOVA.

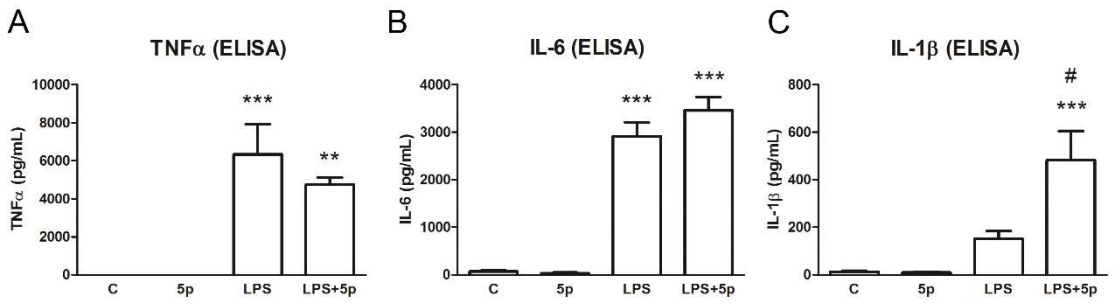

Figure 7. Effects of MSPs on glial proinflammatory cytokine protein levels. Mixed glial cultures treated for 24 h with vehicle (C), MSPs (5p), 100 ng/mL LPS (LPS) or LPS + MSPs (LPS+5p). Protein levels of the proinflammatory cytokines TNFα (A), IL-6 (B) and IL-1β (C) were analysed in the conditioned media by ELISA. Data are shown as mean + SEM, n=5-6 independent cultures. ** $p<0.01$, *** $p<0.001$ vs control. # $p<0.05$ vs LPS. One-way ANOVA.

Supplementary File 1

Time-lapse imaging of mixed glial cultures incubated with rhodamine-B-labelled MSPs (10p). The video shows a sequence of 5h 16min composed of images taken every 2 min and photographed under bright field and red fluorescence microscopy. Internalization of MSPs by rapidly moving refringent microglial cells is observed.