Shedding of Microvesicles from Microglia Contributes to the Effects Induced by Metabotropic Glutamate Receptor 5 Activation on Neuronal Death

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Metabotropic glutamate (mGlu) receptor 5 is involved in neuroinflammation and has been shown to mediate reduced inflammation and neurotoxicity and to modify microglia polarization. On the other hand, blockade of mGlu5 receptor results in inhibition of microglia activation. To dissect this controversy, we investigated whether microvesicles (MVs) released from microglia BV2 cells could contribute to the communication between microglia and neurons and whether this interaction was modulated by mGlu5 receptor. Activation of purinergic ionotropic P2X7 receptor with the stable ATP analog benzoyl-ATP (100 µM) caused rapid MVs shedding from BV2 cells. Ionic currents through P2X7 receptor increased in BV2 cells pretreated for 24 h with the mGlu5 receptor agonist CHPG (200 µM) as by patch-clamp recording. This increase was blunted when microglia cells were activated by exposure to lipopolysaccharide (LPS; 0.1 µg/ml for 6 h). Accordingly, a greater amount of MVs formed after CHPG treatment, an effect prevented by the mGlu5 receptor antagonist MTEP (100 µM), as measured by expression of flotillin, a membrane protein enriched in MVs. Transferred MVs were internalized by SH-SY5Y neurons where they did not modify neuronal death induced by a low concentration of rotenone (0.1 µM for 24 h), but significantly increased rotenone neurotoxicity when shed from CHPG-treated BV2 cells. miR146a was increased in CHPG-treated MVs, an effect concealed in MVs from LPS-activated BV2 cells that showed per se an increase in miRNA146a levels. The present data support a role for microglia-shed MVs in mGlu5-mediated modulation of neuronal death and identify miRNAs as potential critical mediators of this interaction.

Keywords: microglia, miRNA, extracellular vesicle, mGlu5, neuroinflammation, CHPG

INTRODUCTION

Metabotropic glutamate (mGlu) receptors are G protein coupled receptors involved in a variety of functions and processes at the central nervous system (CNS) including synaptic plasticity, neurodevelopment, neuronal excitability, pain, addiction, neurodegeneration, neuroinflammation (Conn and Pin, 1997; Nicoletti et al., 2011). Among the three classes of mGlu receptor subtypes,
group 1 mGlu, which includes mGlu1 and mGlu 5 receptors, is highly expressed throughout the brain (Martin et al., 1992; Romano et al., 1995) and mGlu5 receptor shows high density also in the spinal cord (Berthele et al., 1999). They are both Gq-coupled receptors, thus their activation causes increase of intracellular Ca\(^{2+}\) and predominate at post-synaptic level where they participate to the modulation of synaptic excitability (Shigemoto et al., 1993; Lujan et al., 1996; Biber et al., 1999). Although initial studies made questionable the function of mGlu5 receptor with a dual role, either neuroprotective or neurotoxic, depending on neuronal type and experimental conditions, it is now clearer that mGlu5 receptor interacts with NMDA receptors and likely exerts a permissive role in NMDA excitotoxicity. Hence, several antagonistic modulators of mGlu5 receptor activity exhibit neuroprotective properties whereas mGlu5 receptor agonists are neurotoxic (Parmentier-Batteur et al., 2014). Among others, mGlu5 receptors are not only expressed in neurons, but also in glial cells (Biber et al., 1999). Interestingly, in the cortex, expression of this receptor subtype is even higher in astrocytes than in neurons (Zhang et al., 2014) and appears upregulated in reactive astrocytes (Aronica et al., 2000; Ferraguti et al., 2001; Dolan et al., 2003; Ribeiro et al., 2017). Cultured microglia also express mGlu5 receptor, and its activation has been reported to be associated with reduced inflammation and neurotoxicity (Byrnes et al., 2009) as well as changes in microglia polarization (Loane et al., 2014). However, again, anti-inflammatory effects secondary to activation of mGlu5 receptor are rather controversial as opposing results with reduction of microglia activation by blockade of group 1 mGlu receptors have also been reported (Hsieh et al., 2012). Surprisingly, mGlu5 receptor has also been suggested to act as an alternative binding site for lipopolysaccharide (LPS), thus mediating activation of microglia toward an inflammatory phenotype (Liu et al., 2014).

Microglia affects neuronal function by the release of several factors, but in more recent years an additional way of intercellular communication has been described in several cell types, including glial cells. Extracellular vesicles, either as microvesicles (MVs) or exosomes that differ in size, content and origin, form under basal conditions, or as in the case of microglia, upon stimulation of the purinergic P2X7 receptor. In particular, microglial-derived MVs, ranging from 100 to 1000 nm in diameter, are shed from the extracellular membrane and play as a cargo for cytokines, RNA, miRNA, etc., to neighboring cells. Although the precise kinetics of this interaction are not completely known, shedding of MVs from microglia is a well-established event (Turola et al., 2012; Prada et al., 2013), reported to mediate neurotoxicity by transferring and propagation of pro-inflammatory cytokines (Bianco et al., 2005), or through modulation of β-amyloid aggregation status (Joshi et al., 2014). Even more, MVs can be detected and are increased in the cerebrospinal fluid of humans and rodents under conditions of brain inflammation, suggesting their role in the development of the disease, by spreading the inflammatory process (Verderio et al., 2012).

On these bases, we decided to investigate whether MVs could take part to the effects elicited by mGlu5 receptor activation in microglia and whether these alternative means of interaction between cells could account for the controversial response observed following activation of this receptor.

**MATERIALS AND METHODS**

**Drugs and Reagents**

Cell culture plastics were provided by BD Falcon (Milan, Italy). Media, media supplements, serum, trypsin, and antibiotics, were from Invitrogen Srl (Milan, Italy). (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) Sodium Salt, 3,5-dihydroxyphenylglycine (DHPG), and 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP), were from Tocris (North Point, United Kingdom). 2′(3′)-O-(4-Benzoylbenzoyl)adenosine-5′-triphosphate tri(triethylammonium) salt (Bz-ATP), LPS, rotenone were from Sigma–Aldrich (St. Louis, MO, United States). The following primary antibodies were used: rabbit anti-mGlu 5 (1:500; Millipore, Billerica, MA, United States), rabbit anti-flotillin (1:500; Santa Cruz Biotechnology, CA, United States); goat anti-tumor necrosis factor (TNF) α, (1:100; Santa Cruz Biotechnology), FITC-conjugated mouse anti-TNFα; (1:10; Miltenyi Biotec, Macs, Berisc-Gladbach, Germany).

**Cell Cultures**

Immortalized murine microglial BV2 cells were cultured at 37°C, with 5% CO\(_2\), in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml–100 µg/ml). For the experiments cells were plated in 35 mm tissue culture dishes (200 k/each). The human neuroblastoma SH-SY5Y cell line was cultured at 37°C with 5% CO\(_2\), in DMEM/F-12 supplemented with 10% FBS and penicillin/streptomycin (100 U/ml–100 µg/ml). Cells were plated in 35 mm culture dishes (300 k/each) or in 96 well plates (60 k/well). To achieve BV2 cells conditioned medium (CM), BV2 cells were exposed to either vehicle, CHPG (200 µM), LPS (0.1 µg/ml) or their combination, for 24 h, then washed and incubated with fresh medium for further 6 h. This particular paradigm was chosen in order to allow exposure to treatments for a reasonable time to induce effects before removal so that transferred CM did not contain any drug.

**Shedding of MVs**

BV2 cell cultures were treated with CHPG (200 µM, for 24 h), the selective mGlu5 receptor antagonist MTEP (100 µM added 30 min before CHPG), and LPS (0.1 µg/ml, for 6 h). They were then stimulated with Bz-ATP 100 µM, for 20 min at 37°C in Krebs-Ringer-buffer (Sigma–Aldrich) and the supernatant was subjected to differential centrifugations at 4°C: 5 min at 300 × g to discard cells and debris (P1 pellet); 20 min at 1200 × g to obtain P2 vesicle fraction (P2); 60 min at 10000 × g to obtain vesicle population (P3). The resulting pellet corresponding to MVs (P3) was resuspended in 30 µl of protein solubilisation buffer MPER (Invitrogen srl) supplemented with protein inhibitors and loaded for gel separation by western blotting analysis. For this specific experiment, the total amount of protein from 8 × 35 mm dishes
was resuspended in a small volume and loaded into the gel in order to assess the whole flotillin, expression of MVs formation. Alternatively, MVs were processed for microRNA extraction or resuspended in DMEM/F12 medium and transferred on SH-SY5Y cells. In the latter case four 35-mm dishes per treatment group were used and, after centrifugation, resuspended in a final volume of 1200 µl that were then divided into eight wells of a 96-well multiwell plate (150 µl/well). To achieve experimental consistent conditions, MVs were collected from BV2 cells plated and grown always at the same density and transferred on SH-SY5Y cells also maintained under constant conditions.

Western Blot Analysis

Protein extraction was performed using the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Grand Island, NY, United States) and protein concentration was determined with the Bradford reagent (Sigma–Aldrich). Forty to 50 µg of protein extract for each sample were diluted in loading buffer, denatured by boiling at 95°C for 5 min. In the case of proteins extracted from MVs, an equal volume of the extracts was subjected to electrophoresis. Polyacrylamide-SDS gel at 10% or 4–15% were used. The proteins were transferred on SH-SY5Y cells also maintained under constant conditions.

Cell Viability Assay

SH-SY5Y cell cultures were exposed to rotenone in the presence of either BV2-derived CM or MVs derived from BV2-treated cells. To test SH-SY5Y cell culture viability, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay was performed after 18 h treatments. Cells were exposed to MTT solution (5 mg/ml) for 90 min at 37°C. DMSO was added to dissolve formazan crystals. The absorbance was measured at 540 nm using Varioskan TM LUX multimode microplate reader (ThermoFisher, Milan, Italy).

Flow Cytometry

After treatments BV2 cells were harvested, fixed in PFA 4% and then washed and centrifuged at 300 x g for 10 min. Cells were incubated for 20 min at RT with 300 µl of Inside Stain Kit (Miltenyi Biotec) and then stained with FITC-conjugated anti-TNFα (1:10, Miltenyi Biotec) according to the manufacturer's instruction for 15 min, at RT. Cells were then washed and resuspended in 20 µl of PBS. Analyses were carried out using Amnis® imaging flow cytometer (Millipore).

RNA Extraction

RNA extraction from BV2-derived MVs was performed using the RNeasy Micro Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction. About 20 ng of RNAs were used for reverse transcription (RT) to obtain miRNA-specific cDNAs by using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Scientific), according to the manufacturer's instructions. Four-fifths of the cDNA total volumes were analyzed with quantitative real-time polymerase chain reaction (RT–PCR) using TaqMan MicroRNA Assays (Thermo Scientific). RT–PCRs were performed on a 7900 HT Fast Real Time PCR System (Applied Biosystem – Thermo Scientific), by using TaqMan™ Universal Master Mix II, no UNG (Thermo Scientific), according to the manufacturer's instructions. SnRNA U6 was used as reference gene. Expression fold changes were calculated by the $2^{-\Delta\Delta C_T}$ method.

Reactive Oxygen Species Determination

Determination of reactive oxygen species (ROS) was performed using the 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) assay (Sigma–Aldrich). SH-SY5Y cell cultures, plated in 96-well plates and treated for 24 h as described in the result section, were loaded with carboxy-H2DCFDA at a final concentration of 1 µM and incubated for 30 min in the dark at 37°C, with 5% CO2. After dye's removal, intracellular fluorescence (excitation 485 nm, emission 530 nm) was measured using Varioskan TM LUX multimode microplate reader. Results are given as fluorescence intensity normalized for the number of viable cells as measured by MTT assay.

Whole-Cell Patch-Clamp Recordings and Analysis

Cells plated in 35 mm dishes were transferred from cell culture medium to an extracellular recording solution containing (in mM) the following components: 138 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, at pH 7.25 with NaOH. Borosilicate patch pipette were pulled with P-1000 puller (Sutter Instruments, United States) and resistance was 3–5 MΩ when filled with an intracellular solution containing (in mM): 115 K+ gluconate, 20 KCl, 2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 GTP-Na2, pH 7.25 adjusted with KOH. P2X7 receptor current was recorded in a low divalent cation extracellular solution to avoid blocking action of Ca2+ and Mg2+. This solution contained (in mM): 138 NaCl, 4 KCl, 0.1 CaCl2, 10 glucose, 10 HEPES at pH 7.25 with NaOH. Bz-ATP (100 µM) was added to this solution and application and removal of P2X7 receptor selective agonist and other drugs (as indicated in figure legends) were made locally by a gravity-driven multibarrel local perfusion system operated by solenoid valves (ValveBank II, Automated Scientific, United States) and controlled by computer. Exchange times were measured by the change in junction currents at the tip of an open patch pipette and were estimated in 126.2 ± 9 ms. Experiments were performed at RT using an...
FIGURE 1 | Metabotropic glutamate (mGlu5) receptor mediates anti-inflammatory effect on BV2 microglial cells. Western blot analysis of expression of mGlu5 receptor in BV2 cells treated with LPS (0.1 µg/ml) for 24 h (A). BV2 cells were activated with LPS (0.1 µg/ml for 6 h) after pretreatment with CHPG (200 µM for 24 h) and the selective mGlu5 receptor antagonist MTEP (100 µM added 30 min before CHPG). TNFα expression was evaluated by western blot (B) and flow cytometry (C,D). More specifically, assessment of the percentage of TNFα-positive cells (C) and the level of fluorescence expressed as mean fluorescent intensity (MFI; D) are shown. A representative image acquired by imaging flow cytometry showing cells in their shape and size (brightfield; BF), complexity (side scatter; SS), and TNFα immunostaining (green) is also reported (E). MTT assay on SH-SY5Y cells exposed to rotenone (100 nM for 24 h) in the presence of conditioned medium (CM) from BV2 cells activated with lipopolysaccharide (LPS; 0.1 µg/ml for 6 h) after pretreatment with CHPG (200 µM for 24 h) (F). Student’s t-test (A) and one-way ANOVA followed by Newman–Keuls test for significance (B–E) were applied. *p < 0.05 vs. C; **p < 0.05 vs. LPS (Vhl); §p < 0.05 vs. CHPG+LPS; ◦p < 0.05 vs. CM rot.

EPC7 Plus amplifier (HEKA Elektronik, Germany) in voltage-clamp configuration (holding potential, −70 mV). Data were acquired at 5 kHz and filtered at 1 kHz using a 7-pole Bessel filter and digitized with a low noise data acquisition system, Digidata 1440A (Molecular Devices, United States). Data were recorded and analyzed in pClamp 10 (Molecular Devices) and peak current was normalized to membrane capacitance (Cm) using Microsoft Excel.
**Statistical Analysis**

All data are presented as mean ± SEM and are always derived from at least three independent experiments. Data were analyzed by Student’s *t*-test or, where appropriate, one-way ANOVA followed by Neuman–Keuls test for significance. Plots, bar diagrams and statistical analysis were finalized with GraphPad Prism 5 (GraphPad Software, United States).

**RESULTS**

Expression of mGlu5 receptor in microglial BV2 cells was examined by Western blot analysis. As shown in Figure 1A, microglial BV2 cells expressed mGlu5 receptor and the protein levels were not modified by activation of BV2 cells after exposure to LPS (0.1 µg/ml) for 24 h. The antiinflammatory effect mediated by mGlu5 receptor was confirmed by assessment of TNF-α expression by Western blot and flow cytometry. Treatment for 24 h with the selective mGlu5 receptor agonist CHPG (200 µM) and exposure to LPS (0.1 µg/ml) during the last 6 h, significantly counteracted the LPS-induced increase of TNF-α expression as by Western blot (Figure 1B) and by flow cytometry (Figures 1C–E) analyses. This specific time point (6 h LPS treatment) was chosen based on published data showing that a significant increase of proinflammatory cytokines (Minogue et al., 2012) and of FoxP3 (Chung et al., 2010) is observed within 6 h following LPS exposure in primary microglia and in BV2 cells, respectively. Specificity of this response was shown by prevention of the effect by the mGlu5 receptor antagonist, MTEP (100 µM), added 30 min before the agonist (Figures 1C,D). Changes induced by mGlu5 receptor stimulation in microglial cells modified the response of SH-SY5Y cells to a non-specific toxic insult such as the mitochondrial electron transport inhibitor, rotenone. Transferring of CM from CHPG-pretreated BV2 cells significantly increased SH-SY5Y neuronal death induced by rotenone (100 nM for 24 h; Figure 1F). This effect was observed only with CM from quiescent microglia, but not after activation by exposure to LPS (0.1 µg/ml).

In order to dissect the large discrepancies existing in literature regarding the real role of mGlu5 receptor in neuroinflammation, we decided to investigate whether modulation of mGlu5 receptor could also affect shedding and content of MVs involved in the crosstalk between microglia and neurons. To this purpose, BV2 cells were exposed to the ATP stable analog Bz-ATP (100 µM), a P2X7 receptor agonist, for 20 min, a condition that causes blebbing of cell membrane and ensuing formation of MVs (Antonucci et al., 2012).

Before assessment of MVs formation, patch-clamp recording was performed in order to test purinergic current through P2X7 receptors. BV2 cells exposed to vehicle or CHPG (200 µM) for 24 h were stimulated with Bz-ATP (100 µM) for 3 s in a solution with low divalent ions (0.1 mM Ca²⁺, 0 mM Mg²⁺) to avoid blocking action on channels (Norenberg et al., 2010). Currents elicited by P2X7 receptor activation were significantly increased in BV2 cells pre-treated with CHPG (Figure 2A, upper panel and Figure 2B). In contrast, when BV2 cells were activated by exposure to LPS for 6 h, pre-treatment with CHPG (200 µM, for 24 h) did not elicit any significant increase in current (Figure 2A, lower panel and Figure 2B).
LPS per se was not able to modify P2X7 currents as well (Figure 2A, lower panel and Figure 2B). Membrane capacitance (Cm) showing no change in cell size following treatments is reported (Figure 2C).

Microvesicles shedding from BV2 cells was monitored by time-lapse microscopy following staining of cell membranes with the fluorescent dye FM1-43 (10 µM for 5 min). MVs formation following addition of Bz-ATP (100 µM) was monitored with time-lapse microscopy up to 20 min. Representative images are shown in (A). Western blot of flotillin of equal volume of MVs’ protein extracts from BV2 cells treated with CHPG (100 µM for 24 h) and labeled with the fluorescent dye FM1-43 (10 µM) is shown in (D). Representative images of MVs shed from FM1-43-stained BV2 cells, transferred on top of SH-SY5Y cultures (D). Nuclei were stained with DAPI. Magnification = 40× (A), 63× (D). Scale bars = 20 µm (A) and 10 µm (D).

Metabotropic glutamate 5-receptor activation increases microvesicles (MVs) release from BV2 microglial cells. BV2 cells were treated with DHPG (100 µM for 24 h) and labeled with the fluorescent dye FM1-43 (10 µM for 5 min). MVs formation following addition of Bz-ATP (100 µM) was monitored with time-lapse microscopy up to 20 min. Representative images are shown in (A). Western blot of flotillin of equal volume of MVs’ protein extracts from BV2 cells treated with CHPG (200 µM for 24 h) and MTEP (100 µM for 24 h; B; or with LPS (0.1 µg/ml for 6 h; C). Representative images of MVs shed from FM1-43-stained BV2 cells, transferred on top of SH-SY5Y cultures (D). Nuclei were stained with DAPI. Magnification = 40× (A), 63× (D). Scale bars = 20 µm (A) and 10 µm (D).

*p < 0.05 vs. control. One-way ANOVA followed by Newman–Keuls test for significance (B) and Student’s t-test (C) were applied to detect statistically significant differences.

To assess the effect of microglia-derived MVs on the viability of neuronal cultures, rotenone was applied to SH-SY5Y cells for 24 h and viability was analyzed by MTT assay. Rotenone, at the low concentration used, (0.1 µM for 24 h) induced about 25–30% neuronal death, either alone or in the presence of MVs from untreated BV2 cells (Figure 4A). However, when MVs were from CHPG- or DHPG-treated BV2 cells, a significant increase of rotenone-induced death was observed (Figure 4A). This effect was prevented by pre-incubation (30 min) with the selective mGlu5 receptor antagonist MTEP (100 µM) (Figure 4A). To analyze whether MVs could interfere with the ability of rotenone to induce oxidative stress, ROS formation was assessed in SH-SY5Y cells exposed to rotenone in the presence of MVs shed from BV2 cells either untreated or exposed to CHPG. In the latter condition, ROS generation appeared significantly increased when compared to rotenone challenge in the presence of MVs from control BV2 cells (Figure 4B). Interestingly, potentiation of rotenone toxicity observed in the presence of MVs from CHPG-treated cells was not observed in the presence of MVs shed from LPS-treated microglia (Figure 4C). Searching for cellular factors mediating the observed effects and transferred through shed MVs to the neuronal culture, attention was focused on cytokines. Specifically, we tried to detect TNF-α and interleukin-6 in protein extracts from MVs. However, under the experimental conditions used in the present study, no cytokine was measurable, consistently, in MVs protein extracts by Western blot or by flow cytometry (not shown). In contrast, miRNA could be isolated and, following an initial screening, our preliminary results identified miR146a as a potential mediator of the observed effects. Its expression appeared in fact significantly modified in MVs from CHPG-treated BV2 cells. More specifically, exposure to CHPG increased the expression of miR146a in BV2 cell-shed MVs (Figure 5A), an effect partially counteracted by pretreatment with MTEP (Figure 5B). This effect was significantly modified in LPS-stimulated conditions (0.1 µg/ml for 4 h), where CHPG did not affect the increase of miR146a expression induced by LPS itself (Figure 5A).
FIGURE 4 | Microvesicles shed from CHPG-treated microglia potentiate rotenone-induced neurotoxicity. In (A), MTT assay on SH-SY5Y cells exposed to rotenone (100 nM for 24 h) in the presence of MVs derived from BV2 cells treated with CHPG (200 µM for 24 h), DHPG (100 µM for 24 h), and MTEP (100 µM for 24 h) and transferred to SH-SYSY cells after rotenone (100 nM for 24 h) exposure. Determination of ROS levels in SH-SYSY cells exposed to rotenone (100 nM for 24 h) in the presence of MVs derived from CHPG-treated BV2 cells (B), MTT assay on SH-SYSY cells (100 nM for 24 h) in the presence of MVs from LPS-activated BV2 cells (0.1 µg/ml for 6 h) pretreated with CHPG (200 µM for 24 h). C*: p < 0.05 vs. control; **p < 0.05 vs. rot, *p < 0.05 vs. DHPG vhl. One-way ANOVA followed by Newman–Keuls test for significance was applied.

DISCUSSION

The present study was undertaken with the primary aim to elucidate the role of mGlu5 receptor in neuroinflammation and its modulatory role in microglia. To this purpose, we used BV2 cells, that, for many aspects including gene transcription, protein production and functional attitude, are superimposable to cultured primary microglia, even under activated conditions that mimic an inflammatory state (Henn et al., 2009; Orihuela et al., 2016). The use of a cell line allows also availability of a great amount of cells, hardly warranted by microglia pure cultures. In addition, looking for MVs, it was crucial to deal with a homogeneous cell population and pure cultures of microglia, although highly enriched (90–95% purity), never reach 100% purity and contaminating astrocytes are also a source of MVs.

The BV2 cell line expresses functional mGlu5 receptors (Loane et al., 2009) and has already been used for the study of the signaling pathway activated by this receptor (Chantong et al., 2014). mGlu5 receptor appears the only group 1 mGlu receptor expressed in microglia in vivo and also present in microglia cultures. In contrast, mGlu1 receptor is lacking or barely detectable in cultured microglia (Byrnes et al., 2009), although it has been detected in selected microglia cell populations in multiple sclerosis brain (Klaver et al., 2013). Activation of mGlu5 receptors in primary microglia or in BV2 cell cultures has already been shown to exert anti-inflammatory effect, as revealed by reduction of LPS-induced NO and ROS production, TNFα release, and microglial proliferation (Byrnes et al., 2009; Loane et al., 2009).

In our conditions, we confirmed an anti-inflammatory role for mGlu5 receptor following LPS activation, as suggested by the reduction of the pro-inflammatory cytokine TNFα, by western blot and flow cytometry. However, this anti-inflammatory effect did not produce any neuroprotective outcome, as transferred CM to neuronal cultures was not able to modify neuronal death induced by a low concentration of rotenone. A slight increase of rotenone toxicity was rather observed in neurons exposed to CM from CHPG-treated BV2 cells. This appears in contrast with previous reports showing that CHPG could prevent the neurotoxic effect obtained by co-culturing LPS-activated microglia with neurons (Byrnes et al., 2009). Much higher concentrations of CHPG (20 fold higher) were, however, needed to induce neuroprotection against LPS-stimulated BV2 cell toxicity (Byrnes et al., 2009). Hence, our results indicate the ability of mGlu5 receptor to modify the inflammatory phenotype induced by LPS exposure without, however, any positive outcome on neuronal viability.

Despite the literature and our own observation that indicate anti-inflammatory effect mediated by mGlu5 receptor, when attention is turned to neuropathological conditions such as Alzheimer’s (AD) and Parkinson (PD) diseases, in which activated microglia accumulate around brain lesions and play a role in the neurodegenerative process, the scenario dramatically changes. In vivo studies do not support in fact a protective effect mediated by mGlu5 receptor (Liu and Hong, 2003; Town et al., 2005). More specifically, activation of mGlu5 receptor has been linked to increased synaptotoxicity induced by
the increased response induced by CHPG, a greater amount of
the modified sensitivity to CHPG-induced effect. According to
P2X7-mediated responses are related (reviewed in Kettenmann
this different behavior, but it is known that microglial LPS- and
observed. From our data, we cannot derive any explanation for
enough to let us appreciate such changes in MVs production
of MVs, indirectly measured by the expression of flotillin in
CHPG treatment is able to induce shedding of a greater amount
microglia cells in CHPG-stimulated conditions. Accordingly,

glial cells (Li et al., 2011), is up-regulated by pro-inflammatory
signals (Lukiw et al., 2008) and negatively controls several target
genes involved in the inflammatory response (Lukiw et al.,
2012; Prada et al., 2013), thus enforcing the neuroinflammatory
process (Colombo et al., 2012). We could not observe any change
in rotenone-induced neuronal damage in the presence of MVs
and this may be related to the amount of transferred MVs or
to the ratio MVs:neurons achieved in our conditions. However,
when MVs were derived from CHPG-treated microglia, toxicity
induced by rotenone was significantly increased, suggesting again
that MVs-mediated glia-to-neuron signal can be amplified by
stimulation of mGlu5 receptor in microglia.

miRNA are among factors that have gained, and are expected
to yield, increasing attention for their role in glia to neuron
communication (Su et al., 2016). miRNA transferred through
extracellular vesicles, either from microglia or astrocytes, have
been shown to affect neuronal viability (Hu et al., 2012; Saba et al.,
2012; Mao et al., 2015; Tang et al., 2016; Coleman et al., 2017).
We focused our attention on miR146a that is highly expressed in
glial cells (Li et al., 2011), is up-regulated by pro-inflammatory
signals (Lukiw et al., 2008) and negatively controls several target
genes involved in the inflammatory response (Lukiw et al.,
2008; Li et al., 2011). In addition, transfection with miR146a mimics
has been shown to promote apoptosis in SK-N-SH neurons (Li
et al., 2017). We here report that stimulation of mGlu5 receptor
in BV2 microglia cells up-regulates expression of miR146a that,
upon P2X7 receptor stimulation, can be packaged into shed
MVs and diffuse to neighboring cells. Our data are rather
preliminary and further studies are needed to identify the real
significance of miR146a up-regulation in MVs from CHPG-
treated microglia. Interestingly, also LPS-stimulated microglia
up-regulates miRNA146a in MVs and, once again, under these

β-amyloid peptide in AD and is reported to exert detrimental
effect in animal models of PD (reviewed in Bruno et al.,
2017). All this large body of evidence suggests that the anti-
inflammatory effect mediated by mGlu5 receptor on microglia
may be vanished by effect of drugs acting on this receptor
directly on neurons or that it may be not strong enough or
contrasted by other factors to elicit any protective effect on
neurons. In an attempt to elucidate this aspect, we tried to
investigate the mGlu5-mediated modulation of MVs that have
recently gathered increasing attention for their function as an
alternative/additional way of cell-to-cell and, as in this case,
glia-to-neuron communication (Turola et al., 2012; Verderio
et al., 2012; Frühbeis et al., 2013; Prada et al., 2013; Tetta
et al., 2013; Carandini et al., 2015; Cocucci and Meldolesi,
2015). MVs are shed from microglia upon activation of P2X7
receptor with the stable receptor agonist Bz-ATP (Turola et al.,
2012; Prada et al., 2013). Our results show that the current
elicited by P2X7 receptor activation in microglia is significantly
increased following stimulation of mGlu5 receptor with CHPG.
This effect is in line with the reported potentiation of NMDA-
evoked currents in selected neuronal populations (Awad et al.,
2000; Yang et al., 2011) and suggests a greater response of
microglia cells in CHPG-stimulated conditions. Accordingly,
CHPG treatment is able to induce shedding of a greater amount
of MVs, indirectly measured by the expression of flotillin in
CHPG-derived MVs, when equal volumes of proteins were
loaded. Our microscopic observation instead was not accurate
enough to let us appreciate such changes in MVs production
by eye. Of note, LPS-activated microglia behaved differently,
as no potentiation of NMDA-evoked currents by CHPG was
observed. From our data, we cannot derive any explanation for
this different behavior, but it is known that microglial LPS- and
P2X7-mediated responses are related (reviewed in Kettenmann
et al., 2011) and this interdependence can probably account for
the modified sensitivity to CHPG-induced effect. According to
the increased response induced by CHPG, a greater amount of
MVs is formed and, when transferred to neuronal cultures, the
toxic effect induced by rotenone, either as cell viability or as ROS
formation, is increased.

Microglial-derived MVs have been reported to carry the pro-
inflammatory cytokine interleukin-1β (Bianco et al., 2005) that
may mediate the interplay with other microglia and neurons to
spread and propagate the inflammatory reaction (Verderio et al.,
2012; Prada et al., 2013), thus enforcing the neuroinflammatory
process (Colombo et al., 2012). We could not observe any change
in rotenone-induced neuronal damage in the presence of MVs
and this may be related to the amount of transferred MVs or
to the ratio MVs:neurons achieved in our conditions. However,
when MVs were derived from CHPG-treated microglia, toxicity
induced by rotenone was significantly increased, suggesting again
that MVs-mediated glia-to-neuron signal can be amplified by
stimulation of mGlu5 receptor in microglia.

![FIGURE 5](image_url)

**FIGURE 5** | CHPG increases miRNA146a levels in BV2 cells-shed MVs. BV2 cells were activated with LPS (0.1 µg/ml for 6 h) after pretreatment with CHPG (200 µM, total exposure 24 h). MVs were isolated, miRNA extracted and levels of miRNA146a were analyzed by real time PCR (A). In (B), levels of miRNA146a were analyzed in MVs derived from MPTEP pretreated-cells (100 µM added 30 min before CHPG). Results are expressed as percentage of CHPG. One-way ANOVA followed by Newman–Keuls test (A) and Student's t-test (B) were applied for significance. *p < 0.05 vs. control (A), *p < 0.05 vs. CHPG (B).
activated conditions, the effect of CHPG is blunted. Since this up-regulation is accompanied by different effects on neuronal death induced by CHPG- and LPS-shed MVs, we have to take into account that a greater number of MVs is formed upon CHPG stimulation and that, together with miR146a, other miRNAs or other MVs components can be responsible for changes in the fate of neuronal cells in response to rotenone. In addition, the similar response induced by mGlu5 receptor agonist and LPS points out the common pathway that is potentially activated, suggesting that mGlu5 receptor may function as a mediator of inflammatory processes in microglia, as already reported (Liu et al., 2014).

In our opinion, at least four different conclusions can be drawn from our results. First of all, we confirmed the modulatory role of mGlu5 receptor on neuroinflammation that, however, does not have an obvious, ensuing influence on neuronal survival. Even more important, these effects may involve not only conventional interplay between glial cells and neurons, but also novel mediators represented by MVs shed from microglia that function as a cargo for neuromodulators. Third, in our experimental conditions, MVs are formed after stimulation of ATP and shedding and content of MVs seem to vary depending on microglia level of activation. Finally, miRNA transferred through MVs may contribute to the interaction between microglia and neurons thus representing a novel, targetable mediator of neuroinflammation.

AUTHOR CONTRIBUTIONS

MB, SFS, SM, PP performed all the experiments, collected, and analyzed all data. MC performed the electrophysiological studies. MR, MP carried out the miRNA studies. FN contributed to extension and revision of the ms. MAS designed the experiments and wrote the manuscript with the contribution of all other authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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