

# mGlu3 receptor regulates microglial cell reactivity in neonatal rats

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## Research

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## Abstract

**Background:** Perinatal inflammation is a key factor of brain vulnerability in neonates born preterm or with intra-uterine growth restriction (IUGR), two leading conditions associated with brain injury and responsible for neurocognitive and behavioral disorders. Systemic inflammation is recognized to activate microglia, known to be the critical modulators of brain vulnerability. Although some evidence support a role for metabotropic glutamate receptor 3 (mGlu3 receptor) in modulation of neuroinflammation, its functions are still unknown in the developing microglia.

**Methods:** We used a double-hit rat model of perinatal brain injury induced by a gestational low-protein diet combined with interleukin-1 $\beta$  injections (LPD/IL-1 $\beta$ ), mimicking both IUGR and prematurity-related inflammation. The effect of LPD/IL-1 $\beta$  on mGlu3 receptor expression and the effect of mGlu3 receptor modulation on microglial reactivity were investigated using a combination of pharmacological, histological, and molecular approaches.

**Results:** Exposure to LPD/IL-1 $\beta$  significantly down-regulated *Grm3* gene expression in the developing microglia. Both transcriptomic analyses and pharmacological modulation of mGlu3 receptor demonstrated its central position in the control of inflammation in resting and activated microglia. Microglia hyper-reactivity to inflammatory challenge induced by LPD/IL-1 $\beta$  exposure was reduced by an mGlu3 receptor agonist. Conversely, both specific pharmacological blockade and siRNA knock-down of mGlu3 receptors in control microglia mimicked the pro-inflammatory phenotype observed in microglial cells exposed to LPD/IL-1 $\beta$ .

**Conclusions:** Overall, these data show that *Grm3* plays a central role in the regulation of microglial reactivity in the immature brain. Selective pharmacological activation of mGlu3 receptors may prevent inflammatory-induced perinatal brain injury.

## Background

Every year, 30 million infants worldwide are delivered after intra-uterine growth restriction (IUGR), and 15 million are born preterm (1, 2). These two complications are the leading causes of ante/perinatal stress and brain injury, responsible for neurocognitive and behavioral disorders in more than 9 million children each year. Both prematurity and IUGR are associated with perinatal systemic inflammation, identified to activate microglia (3), the resident macrophages of the central nervous system (CNS), and to be the best predictor of subsequent neurological impairment (4, 5). Microglial cells can acquire distinct phenotypes in response to perinatal stimuli that allow them to not only disrupt developmental processes but also support repair and regeneration. Recently, it was hypothesized that the loss of control of postnatal microglial activity is a precipitating factor for neurodevelopmental disorders from cerebral palsy to autism spectrum disorders (6). The control of microglial activity during this critical period therefore appears to be key in protecting the developing brain.

The level of glutamate, the major excitatory neurotransmitter in the CNS, is finely tuned during the early postnatal period, when the developing brain is highly vulnerable to excitotoxic insults. The action of glutamate on microglia in perinatal life is largely unexplored. Indeed, most studies focusing on the roles of glutamate during the neonatal period have explored neuronal and astroglial cell function (7–13)

Metabotropic glutamate (mGlu) receptors are expressed in mature microglia and modulate microglial functions in physiology and pathology (14). These receptors form a family of eight subtypes, subdivided into three groups on the basis of their amino-acid sequence, pharmacological profile, and G-protein coupling (15). Their dysfunction is commonly observed in the pathophysiology of neurodegenerative and neuropsychiatric diseases (16–20).

Targeting mGlu receptors represents a realistic therapeutic strategy due to the availability of allosteric modulators with long-lasting effects and a better safety profile than pharmacological agents that modulate ionotropic glutamate receptors. Indeed, recent evidence shows positive allosteric modulators of mGlu3 receptors in astrocytes or mGlu4 receptors in oligodendrocytes to be promising pharmacological approaches for the treatment of neurodegenerative disorders (21).

The only published study concerning neonatal microglia reported a role for mGlu5 receptors in the control of neuro-inflammation following excitotoxic insult (7). Recent studies have shown that mGlu3 receptors boost mGlu5 receptor signaling in brain tissue and cultured astrocytes and that mGlu3 activation is required for a full mGlu5 receptor response to agonist activation (22–24). We therefore investigated whether mGlu3 receptors play a role in the modulation of brain inflammation, in particular in microglial cell reactivity.

We investigated *Grm3* gene expression *in vivo* and in primary microglial cells sorted from neonatal rats. Bioinformatics analysis of a large microarray data set shows *Grm3* to be a major hub in the control of inflammation in resting and activated microglia. We also show that targeting mGlu3 receptors drastically modulates microglial activation using pharmacological and molecular approaches.

## Materials And Methods

### Animals, diets, and postnatal inflammatory challenge: LPD/IL-1 $\beta$ rat model

All experiments were carried out in compliance with Inserm ethical rules, approved by the institutional review board (Ministry of Higher Education and Scientific Research, Directorate-General for Research and Innovation, Paris, France), in accordance with the European Communities Council Directive 2010/63/EU. Briefly, Sprague–Dawley dams (Janvier Labs, Le Genest-Saint-Isle, France) were randomly divided into two groups according to their diet: a 22% (normal) protein diet (control, CTRL) or an isocaloric 9% protein diet (LPD) from the day of conception until delivery. On postnatal day 1 (P1) and P2, pups were injected intraperitoneally (i.p.), twice a day, with PBS or IL-1 $\beta$  (20  $\mu$ g/kg, Miltenyi Biotec, Bergisch Gladbach, Germany), as previously reported (25).

### Cortical sample collection and magnetic sorting of microglial cells

Cortical samples were collected from CTRL and LPD/IL-1 $\beta$  pups at P1, P4, P7, P10, and P20. Primary microglial cells were sorted at P1, P4, P7, P10, and P20 using magnetic antibody-based cell sorting (MACS) and CD11b antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously reported (25). The purity of the P4 and P7 MACS CD11b<sup>+</sup> fractions was validated by RT-qPCR performed on the positive and negative CD11b cell fractions for *Itgam*, *Gfap*, *Neun*, and *Mbp* and the arbitrary units normalized to the respective negative fraction in CTRL: P4 CTRL (mean  $\pm$  SEM): *Itgam* 99.78  $\pm$  11.29, *Gfap* 0.13  $\pm$  0.01, *Neun* 0.08  $\pm$  0.02, *Mbp* 0.02  $\pm$  0.003; P4 LPD/IL-1 $\beta$ : *Itgam* 109.15  $\pm$  5.27, *Gfap* 0.11  $\pm$  0.1, *Neun* 0.02  $\pm$  0.002, *Mbp* 0.01  $\pm$  0.01; P7 CTRL: *Itgam* 203.80  $\pm$  9.95, *Gfap* 0.07  $\pm$  0.01, *Neun* 0.04  $\pm$  0.004, *Mbp* 0.06  $\pm$  0.02; P7 LPD/IL-1 $\beta$ : *Itgam* 277.5  $\pm$  23.27, *Gfap* 0.09  $\pm$  0.01, *Neun* 0.05  $\pm$  0.01, *Mbp* 0.02  $\pm$  0.003.

### RNA purification, cDNA synthesis, and real-time qPCR

Samples were immediately snap frozen after collection. Total RNA from the cortex was extracted using Nucleazol reagent and the NucleoSpin RNA Set for NucleoZol (Macherey-Nagel, Hoerdt, France). Microglial RNA was isolated using NucleoSpin RNA Plus XS (Macherey-Nagel). RNA quantity and quality were determined using the Nanodrop<sup>TM</sup> apparatus (ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the Iscript<sup>TM</sup> cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). Primers were designed using Primer3Plus software and sequences are available on request. qRT-PCR was performed in triplicate as reported in (26) using the Ribosomal protein L13 (Rpl13) as the reference gene.

### RNA preparation from sorted microglial cells, cDNA synthesis, microarray hybridization, and bioinformatics analysis

Total RNA was extracted from CTRL and LPD/IL-1 $\beta$  P4 animals and cDNA synthesis and microarray hybridization performed as previously described (25). For microarray hybridization, labeled cRNA was generated using classical protocols for Affymetrix array hybridization. Three points per condition were analyzed by RaGene-2\_0-st microarray hybridization, for which 36,685 probes are examined and directly analyzed using Gene Set Enrichment Analysis (<https://www.gsea-msigdb.org/gsea/index.jsp> (27)). Networks analysis of the microarray data was also performed using String (<https://string-db.org/>). The numerical data from the networks obtained were then exported to Cytoscape (<https://cytoscape.org/>) to identify putative hub genes, using the Network Analysis option. Microarray results were validated as previously reported (25).

### Protein extraction and immunoblotting assay

Total protein was extracted from the cortex and microglial cells sorted from P4 and P7 rats of the CTRL and LPD/IL-1 $\beta$  experimental groups. Cortex samples were homogenized in lysis buffer (0.32 M sucrose, 4 mM HEPES pH 7.4, 1% SDS). Complete, Mini, EDTA-free Protease Inhibitor Cocktail and PhosSTOP (Roche, Meylan, France) were added. RIPA buffer (Sigma-Aldrich, St Quentin Fallavier, France), supplemented with protease and phosphatase inhibitors, was used to extract protein from the microglial cells. Proteins were quantified using the Bradford assay (Sigma-Aldrich) and 30  $\mu$ g from the cortex and 20  $\mu$ g from the microglia re-suspended in Laemmli Sample Buffer (Bio-Rad) with 2.5% 2-mercaptoethanol (Sigma-Aldrich). Samples were separated on 4–15% Mini-PROTEAN TGX Precast Protein Gels and the proteins transferred to nitrocellulose membranes using a Trans-Blot Turbo Mini (Bio-Rad). Proteins were stained using Ponceau S solution (Sigma-Aldrich) and digital images acquired. The optical density of each well was measured using NIH ImageJ medical imaging software. Blots were incubated in a blocking solution containing Tris-buffered saline (TBS), 0.1% Tween-20 (Sigma-Aldrich), and 5% non-fat milk (Bio-Rad) for 1 h at room temperature. Blots were then incubated overnight with rabbit anti-mGlu3 receptor (1:600; AGC-012, Alomone Labs, Jerusalem, Israël) or mouse Anti- $\beta$ -Actin (1:10000; clone AC-74, Sigma-Aldrich) in blocking solution at 4°C. After incubation with the primary antibody, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2000; 0545, Sigma-Aldrich) or horseradish peroxidase conjugated goat anti-mouse (1:2000; 12-349, Sigma-Aldrich), for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence using Clarity Max ECL (Bio-Rad). Digital images were acquired and the optical density of each band measured as described above. The ratio of the target to Ponceau S or  $\beta$ -Actin was then determined and the values compared for statistical significance.

### Drugs

LY 379268, a highly selective group II mGlu receptor agonist (Tocris Bioscience, Rennes, France), was dissolved in sterile water. LY 2389575 hydrochloride, a selective negative allosteric modulator of mGlu3 receptors (Tocris) and Ro 64-5229, selective, non-competitive mGlu2 receptor antagonist (Tocris) were dissolved in DMSO.

### Primary microglial cell culture: real-time qPCR and morphological assay

Microglial cells were isolated from CTRL and LPD/IL-1 $\beta$  animals at P4 and P7 and cultured as previously reported (25). CTRL microglial cells were treated with LY 379268 (0.5, 1, or 5  $\mu$ M) + Ro 64-5229 (25  $\mu$ M) to establish the optimal drug concentration (1  $\mu$ M). After 48 h, CTRL and LPD/IL-1 $\beta$  microglia were divided into four groups: (1) DMSO (0.30%), (2) LY 379268 (1  $\mu$ M) + Ro 64-5229 (25  $\mu$ M), (3) IL-1 $\beta$  (50 ng/ml) + IFN $\gamma$  (20 ng/ml) for 1 h, and (4) LY 379268 (1  $\mu$ M) + Ro 64-5229 (25  $\mu$ M) for 1 h and then IL-1 $\beta$  + IFN $\gamma$  for 4 h.

A second series of experiments was performed on P4 and P7 CTRL microglial cells. CTRL microglial cells were treated with LY 2389575 (1, 3, or 5  $\mu$ M) to establish the optimal drug concentration (5  $\mu$ M). Forty-eight hours after plating, cells were divided into four groups: (1) DMSO (0.30%), (2) LY 2389575 (5  $\mu$ M), (3) IL-1 $\beta$  + IFN $\gamma$  for 1 h, and (4) LY 2389575 (5  $\mu$ M) for 1 h and then IL-1 $\beta$  + IFN $\gamma$  for 4 h.

Total RNA was extracted using NucleoSpin RNA Plus XS. RNA quality and quantity were determined, cDNA synthesized, and real-time qPCR performed as reported above.

Morphological analysis was performed after increasing the drug exposure time to 12 h. After fixation with 4% PFA, cells were stained with goat anti-Iba1 (1:500; ab5076, Abcam, Paris, France) and DAPI (1:10000). Iba1<sup>+</sup> cells were analyzed using a fluorescent microscope (Nikon Eclipse Ti-E): (i) area, (ii) perimeter, and (iii) cell circularity ( $4\pi \times (\text{area}/\text{perimeter}^2)$ ) were determined.

## siRNA experiments

Experiments to knock-down *Grm3* expression were performed on P7 CTRL microglia using Viromer Blue Reagent (Lipocalyx, Halle, Germany) according to the manufacturer's protocol. Transfection efficiency and cell viability after Viromer exposure were evaluated using the siGLO Red Transfection Indicator (25 nM or 50 nM) (Dharmacon, Lafayette, CO, USA). Cells were fixed in 4% PFA, stained with anti-Iba1 antibody, and analyzed by fluorescence microscopy. Cells were treated 48 h after plating with (1) ON-TARGETplus Non-targeting Pool (25 nM or 50 nM) or (2) ON-TARGETplus Rat *Grm3* (24416) siRNA - SMARTpool (25 nM or 50 nM) (Dharmacon) for 4 h. The medium was replaced by fresh culture medium and the cells incubated at 37°C for 48 h before proceeding to RNA and protein extraction.

## Statistical analysis

Statistical analysis of all data was performed using GraphPad PRISM version 8.0 (San Diego, CA, USA). Student's t test was performed for two-group comparisons. A one- or two-way ANOVA, followed by Newman-Keul's post hoc multiple comparison tests, was performed for comparison of more than two groups. A Pearson correlation test was used to analyze relationship between *Grm3* and inflammatory cytokines mRNA expression in siRNA experiment. A multiple t-test was performed to analyze microarray data. Significance was set at  $p < 0.05$  for all tests. Number of samples, statistics, and p values, were reported in Additional Table 1. Morphological data were analyzed by a person who was blind to experimental conditions. Pups from 2-3 litters were used in each experiment.

## Results

### LPD/ IL-1 $\beta$ exposure induces downregulation of *Grm3* gene expression in microglia

We studied the transcriptomic consequences of the combination of gestational LPD and neonatal IL-1 $\beta$  exposure on the glutamatergic system in microglial cells. Bioinformatics analysis performed on microglial cells sorted from P4 rat pups showed consistent significant alterations of the glutamatergic functions in animals subjected to LPD/IL-1 $\beta$  relative to CTRL animals (Fig 1A). Overall, gene sets related to both glutamate-receptor binding and activity were significantly enriched in LPD/IL-1 $\beta$ -exposed animals (Fig 1A, B). Focusing on metabotropic receptors, the microarray analysis showed the mGlu3 receptor-coding gene (*Grm3*) to be the most highly expressed glutamate receptor at P4 in primary microglial cells and the most deregulated among mGlu receptor-coding genes (Fig 1C). *Grm3* was downregulated in sorted microglia, both before plating (Fig 1D) and after two days of cell culture (Fig 1E). Network analysis showed that *Grm3* is a major "hub" gene in a 43-gene network (Fig 1F) and that the network centered on *Grm3* is connected to another network "Cytokine-cytokine receptor interaction/Chemokine signaling pathway", characterized by the genes *Ccl9*, *Ccl6*, *Ccr1*, *Ccr11*. Analysis of gene expression within the same network showed *Grm3* downregulation to be consistently associated with upregulation of these inflammation-related genes (Fig 1G).

We next assessed whether the developmentally regulated expression of *Grm3* during the first three postnatal weeks was altered by LPD/IL-1 $\beta$  exposure. qRT-PCR analysis showed that *Grm3* expression in the cerebral cortex increased from birth to P4/P7, followed by a decline from P10 in CTRL animals. The early postnatal increase in *Grm3* expression was delayed in rats exposed to LPD/IL-1 $\beta$ , showing significantly lower expression of *Grm3* at P4 than in CTRL animals (Fig 2A).

This developmental profile was also observed in microglial cells, with maximum expression at P4 and a long-lasting reduction in *Grm3* levels between P4 and P10 in animals exposed to LPD/IL-1 $\beta$  (Fig 2B). Alterations of *Grm3* gene expression induced by LPD/IL-1 $\beta$  exposure were further confirmed by immunoblot analysis, showing a significant reduction at P4 in both the cortex (Fig 2C) and sorted microglia (Fig 2E). We also observed this reduction at P7 (Fig 2D, Fig 2F).

### *In vitro* mGlu3 receptor activation limits microglial reactivity following LPD/IL-1 $\beta$ exposure

Microglial cells sorted from CTRL rats were stimulated with IL-1 $\beta$  and IFN $\gamma$  and treated with the mGlu2/3 receptor agonist, LY 379268 + Ro 64-5229, a selective non-competitive mGlu2 receptor antagonist, to assess the effect of mGlu3 receptor activation on inflammatory-related gene expression. Addition of IL-1 $\beta$  + IFN $\gamma$  caused a highly significant increase in pro-inflammatory cytokines gene expression and, to a lesser extent, in anti-inflammatory and immunoregulatory cytokines gene expression. Selective activation of mGlu3 receptors by LY 379268 (1 $\mu$ M) largely reduced the transcription of *Il6*, *Tnfa* and *Nos2* genes (Additional Fig 1A), without significant change in the expression of anti-inflammatory and immunoregulatory cytokines (Additional Fig 1B).

We next assessed the effect of pharmacological activation of mGlu3 receptors on microglial reactivity in cultured microglia sorted from P4 animals that had been exposed to LPD/IL-1 $\beta$  and age-matched CTRL rats. *In vivo*, LPD/IL-1 $\beta$  induced changes in the morphology of cultured microglia, which showed an amoeboid shape, characterized by a reduced area, reduced perimeter, and increased circularity (Fig 3A, 3B). *In vitro* inflammatory challenge with IL-1 $\beta$  + IFN $\gamma$  induced changes in the cell morphology of microglial cells sorted from CTRL animals but not that of cells sorted from animals exposed to LPD/IL-1 $\beta$ , which were already activated. The constitutive inflammatory phenotype observed in microglia sorted from rats exposed to LPD/IL-1 $\beta$  was reversed by mGlu3 receptors activation. Morphological changes induced by *in vitro* stimulation with IL-1 $\beta$  + IFN $\gamma$  were also partially prevented by mGlu3 receptor activation (Fig 3A, 3B).

We next examined the effect of mGlu3 receptor activation on the expression of inflammatory markers in microglia sorted from pups exposed to LPD/IL-1 $\beta$  or control microglia, with or without *in vitro* stimulation with IL-1 $\beta$  + IFN $\gamma$ . Microglia from LPD/IL-1 $\beta$ -exposed animals showed high expression of *Il6*, *Tnfa*, and *Nos2* (Fig 3C) and lower expression of *Mrc1* (Fig 3D) under basal conditions. Stimulation with IL-1 $\beta$  + IFN $\gamma$  induced a greater increase in inflammatory marker expression in microglia sorted from LPD/IL-1 $\beta$  rats than in microglia from CTRL rats (Fig 3C, 3D). Pharmacological activation of mGlu3 receptors mitigated the basal hyper-reactivity of microglia sorted from P4 animals exposed to LPD/IL-1 $\beta$  and also reduced the pro-inflammatory effect of IL-1 $\beta$  + IFN $\gamma$  challenge in both CTRL and LPD/IL-1 $\beta$  microglia (Fig 3C).

We performed similar experiments using cultured microglia from P7 animals, in which cells sorted from animals exposed to LPD/IL-1 $\beta$  showed an activated phenotype under basal conditions and a greater pro-inflammatory response to *in vitro* challenge with IL-1 $\beta$  + IFN $\gamma$  (Fig 4A, 4B, 4C). These changes were all reversed or mitigated by pharmacological activation of mGlu3 receptors (Fig 4A, 4B, 4C).

### Pharmacological blockade and knock-down of mGlu3 receptors induces a pro-inflammatory phenotype in microglia

It was important to demonstrate that endogenous blockade of mGlu3 can replicate the pro-inflammatory phenotype observed in microglia sorted from rats exposed to LPD/IL-1 $\beta$  in microglial sorted from control pups to validate mGlu3 receptors as candidate drug targets to alleviate perinatal neuro-inflammation associated with IUGR or prematurity. Our first strategy was to use compound LY 2389575, a negative allosteric modulator of mGlu3 receptors (28). Selective pharmacological blockade of mGlu3 receptors using LY 2389575 exacerbated the effect of *in vitro* inflammatory challenge with IL-1 $\beta$  + IFN $\gamma$  on pro-inflammatory cytokine gene expression (Additional Fig 2A) and conversely significantly reduced the gene expression of the anti-inflammatory cytokine Arg1 (Additional Fig 2B).

According to optimal drug concentration (5  $\mu$ M) conferring the highest effect on inflammatory-related gene expression in microglial cell culture, we next examined the morphology of microglial cells sorted from CTRL pups at P4 and P7 after treatment with LY 2389575 (5  $\mu$ M), with or without IL-1 $\beta$  + IFN $\gamma$  challenge. Pharmacological blockade of mGlu3 receptors induced a change in cell morphology toward an amoeboid shape in both P4 and P7 microglia, with a significant reduction in cell area and perimeter and an increase in cell circularity (Fig 5A, 5B, 5E, 5F).

We further characterized the effect of mGlu3 receptor blockade on microglial reactivity by studying the effect of LY 2389575, with or without IL-1 $\beta$  + IFN $\gamma$  challenge, on the expression of inflammation-related markers in a second series of *in vitro* experiments. Pharmacological blockade of mGlu3 receptors increased the expression of all pro-inflammatory markers in primary microglia from P4 pups under basal conditions and in response to IL-1 $\beta$  + IFN $\gamma$  (Fig 5C). LY 2389575 also induced an increase in *Mrc1* levels under basal conditions and a significant reduction of *Arg1* expression in response to IL-1 $\beta$  + IFN $\gamma$  (Fig 5D). Similar effects were produced by LY 2389575 in microglia sorted from P7 pups (Fig 5G). mGlu3 receptor blockade also reduced the expression of IL-4Ra under basal conditions and in response to the inflammatory challenge (Fig 5H).

We used siRNA as a second strategy to knock-down mGlu3 receptor levels in microglia sorted from CTRL pups. We first tested the transfection efficiency using siGLO Red (25 nM or 50 nM) obtaining a 58.2 % (25 nM) and 83.2% (50 nM) transfection rate 48 h after the application of siGLO Red (Additional Fig 3A, 3B). *Grm3* siRNA (25 nM) slightly reduced *Grm3* expression (Fig 6A) without modifying the level of inflammatory and anti-inflammatory cytokines gene expression (data not shown). In contrast, treatment with *Grm3* siRNA (50 nM) significantly reduced mGlu3 receptor mRNA and protein levels after 48h (Fig 6B, Fig 6C), increased the level of IL-6, *Nos2*, TNF $\alpha$ , *Mrc1*, and IL-4Ra transcripts, and reduced the level of the transcript for the anti-inflammatory cytokine *Arg1* (Fig 6D, 6E). Interestingly, the level of *Grm3* expression was negatively correlated to the expression of IL-6 (Pearson correlation:  $r = -0.7572$ ,  $p < 0.0001$ ), TNF $\alpha$  ( $r = -0.5085$ ,  $p = 0.0132$ ), *Nos2* ( $r = -0.8115$ ,  $p < 0.0001$ ), *Mrc1* ( $r = -0.5302$ ,  $p = 0.0093$ ), and IL-4Ra ( $r = -0.7956$ ,  $p < 0.0001$ ) and positively correlated to *Arg1* expression ( $r = 0.5048$ ,  $p = 0.0166$ ) (Additional Fig 4A, 4B). Overall, these data strongly suggest that mGlu3 receptor blockade or knock-down induces a hyper-activated phenotype in microglia.

## Discussion

Here, we show that the mGlu3 receptor is highly expressed by microglia during early postnatal life and critically regulates microglial reactivity by exerting anti-inflammatory properties. We demonstrate that endogenous activation of microglia mGlu3 receptors limits inflammation and that they respond to pharmacological activation under pathological conditions, thus making them candidate drug targets for therapeutic intervention.

Prematurity and IUGR are associated with perinatal and postnatal inflammation, contributing to cerebral palsy and cognitive and behavioral disorders (1). We developed a preclinical model of perinatal brain injury associated with IUGR by combining gestational LPD (29) with IL-1b injection of the pups. Animals exposed to this double-hit insult showed neuro-inflammation and microglial hyper-activity, detected not only *in vivo* but also in cultured microglia sorted from the neonatal brain (25, 30).

The mGlu3 receptor is unique among all mGlu receptor subtypes because it was the only receptor down-regulated in the microglia of pups exposed to LPD/IL-1b. Because pharmacological blockade or knock-down of mGlu3 receptors mimicked LPD/IL-1b in inducing a pro-inflammatory microglial phenotype, it is reasonable to assume that the down-regulation of mGlu3 receptors is a key modulator in the chain of reactions that cause neuro-inflammation and the ensuing neurological complications associated with IUGR.

The age-dependent profile of *Grm3* expression was not uniform in the cerebral cortex and isolated microglia. LPD/IL-1 $\beta$  reduced mGlu3 mRNA levels in the cerebral cortex at P4, but not at later postnatal days. In contrast, there was a substantial reduction in the level of mGlu3 receptor transcripts in microglia at P4, P7, and P10, but not P20. The more limited down-regulation of mGlu3 levels in the entire cerebral cortex may reflect the presence of the mGlu3 receptor in neurons and astrocytes (15), for which the impact of LPD/IL-1 $\beta$  on receptor expression is yet to be determined. We did not investigate the molecular mechanisms leading to mGlu3 down-regulation here but it is possible that there are transient epigenetic modifications and/or regulation of transcriptional factors that selectively and temporally change receptor expression in microglial cells exposed to LPD/IL-1 $\beta$ . This hypothesis is supported by an increased expression of the RE1-Silencing Transcription factor (REST) observed in LPD/IL-1 $\beta$ -exposed microglia cells. REST is a transcriptional repressor that silences target gene expression via epigenetic remodeling mechanisms (31). Interestingly, it has been shown to bind to the promoter of *Grm3* (32).

There are three distinct temporal stages in microglial development ("early microglia" until embryonic day 14, "pre-microglia" from embryonic day 14 to a few weeks after birth, and adult microglia afterwards). Transition through these stages, which is driven by a stepwise program of gene expression, critically regulates brain homeostasis (33). Alterations of the microglial developmental trajectory during the perinatal period are associated with abnormal cell- and

time-specific gene expression in other neural cell lineages, resulting in altered neuronal development (33, 34). Early exposure to inflammation affects the transition between the maturational stages of microglia, causing a shift towards an advanced developmental stage (33, 35).

Glutamate is a key player in the functional crosstalk between microglia, neurons, and astrocytes, and microglial cells express both ionotropic and metabotropic glutamate receptors (see Introduction and references therein). The role of mGlu3 receptors in the modulation of microglial reactivity has been examined only in a few studies in relation to neurotoxicity associated with typical CNS disorders of adult life, such as Alzheimer's disease and multiple sclerosis (16, 20, 36). We were surprised to find that *Gm3* was the major "hub" gene among several glutamate-related genes in the developing microglia and that it was the only mGlu receptor-encoding gene showing a robust response to an adverse perinatal environment that mimics IUGR and perinatal inflammation. We activated mGlu3 receptors using the orthosteric agonist, LY 379268, which is brain permeant and displays a high affinity for mGlu3 receptors (37). LY 379268 also has nanomolar affinity for mGlu2 receptors, but this bias was limited by the nearly absent mGlu2 expression in the developing microglia (see Fig. 1) and by the use of Ro 64-5229, a negative allosteric modulator of mGlu2 receptors. Activation of mGlu3 receptors by LY 379268 reduced microglial activation in response to the *in vitro* inflammatory challenge and alleviated microglial hyper-activity following LPD/IL-1 $\beta$  exposure from birth to P7. Conversely, pharmacological blockade and knock-down of mGlu3 receptors enhanced microglial reactivity under both basal and activated conditions.

The anti-inflammatory action of microglial mGlu3 receptors contrasts with the pro-inflammatory action of mGlu2 receptors found in various experimental models (20, 38). mGlu3 and mGlu2 receptors share a similar amino-acid sequence, pharmacological profile of activation, and transduction mechanisms. Both receptors are coupled to G<sub>i/o</sub> and their activation inhibits adenylyl cyclase activity in heterologous expression systems (15). We cannot exclude that the inhibition of cAMP formation drives the anti-inflammatory effects in the developing microglia, in which mGlu2 receptors were nearly absent. mGlu3 receptors are different from mGlu2 receptors in that they boost mGlu5 receptor signaling, enhancing mGlu5 receptor-mediated phospho-inositide hydrolysis. Crosstalk between mGlu3 and mGlu5 receptors has been demonstrated in heterologous expression systems, brain slice preparations, cultured astrocytes (22), and cultured microglia (authors' unpublished observation).

A large body of evidence shows that mGlu5 receptor activation drives microglia towards an anti-inflammatory phenotype (39-42) and restrains microglia-driven neuro-inflammation in models of temporal lobe epilepsy (43), Parkinson's disease (44), subarachnoid hemorrhage (45), and traumatic brain injury (46). It is therefore highly possible that the facilitation of mGlu5 receptor signaling is involved in the anti-inflammatory action of mGlu3 receptors in developing microglia. Functional crosstalk between mGlu3 receptors and G<sub>q/11</sub>-coupled oxytocin receptors has also been hypothesized to play a role in the anti-inflammatory effect of oxytocin (25). Further studies using specific G-protein inhibitors or co-stimulation of mGlu3 and other G protein-coupled receptors are needed to better understand the molecular determinants of the anti-inflammatory effects displayed by mGlu3 receptors in the developing microglia.

## Conclusions

In conclusion, we show that the microglial mGlu3 receptor is highly down-regulated in a rat model of perinatal brain injury and its pharmacological modulation a major determinant of microglial activity. The fine-tuning of microglial activation is key for preventing brain damage, because microglia are not only a cornerstone of the brain inflammatory response but also modulate certain functions of the brain environment, including synaptic pruning and maturation (47). Selective pharmacological activation of mGlu3 receptors during early postnatal life might mitigate neuro-inflammation associated with IUGR or other perinatal adverse conditions, thus preventing the development of neurological disorders later in life.

## Abbreviations

- IUGR: intra-uterine growth restriction
- mGlu3 receptor: metabotropic glutamate receptor 3
- LPD: low protein diet
- CNS: central nervous system
- CTRL: control
- MACS: magnetic antibody-based cell sorting

## Declarations

Ethics approval: All experiments were carried out in compliance with Inserm ethical rules, approved by the institutional review board (Ministry of Higher Education and Scientific Research, Directorate-General for Research and Innovation, Paris, France), in accordance with the European Communities Council Directive 2010/63/EU.

Consent for publication: Not applicable.

Availability of data and materials: Raw data reported in the manuscript are archived at the EMBL-European Bioinformatics Institute (EBI) under E-MATB-6631.

Competing interests: The authors have no conflict of interest to declare.

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Authors' contribution: MZ, JM, FN and OB conceived the project and wrote the paper, MZ, JP performed the experiments and the data analysis, DV perform the Bioinformatics analysis. DV, FF, LI, RO contributed to the paper editing.

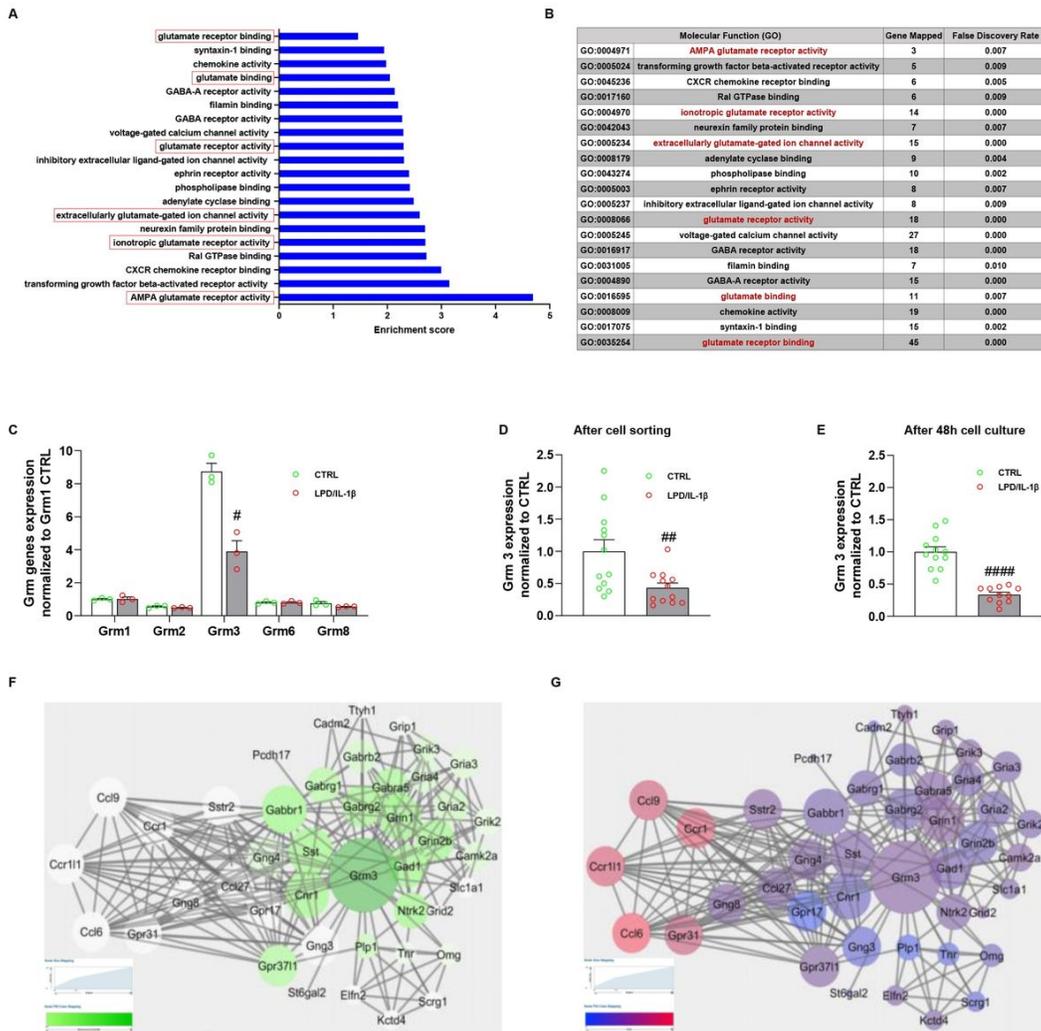
Aknowledgements: Not applicable.

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## Figures



**Figure 1**

Transcriptomic analysis of Grm3 deregulation associated with neonatal neuro-inflammation in rats. (A) Functional network enrichment in microglial cells magnetically sorted from P4 animals under basal conditions (CTRL) and following LPD/IL-1 $\beta$  challenge inducing neonatal neuro-inflammation. The 20 most enriched molecular functions are reported in the graph, sorted by increasing enrichment score. The ontologies associated with glutamate receptor activity are underlined in red. (B) Description of the number of genes mapped and their respective FDR values for each molecular function. In red are reported the gene ontology pathways that were associated with glutamate receptor activity. (C) Grm gene expression in microglia cells sorted from CTRL and LPD/IL-1 $\beta$  animals at P4 according to a Gene Set Enrichment Analysis against the Gene Ontology Database. Data (mean  $\pm$  SEM) are relative to Grm1 expression in CTRL animals. Multiple t-test; # $p < 0.05$ . (D) Grm3 gene expression in microglial cells just sorted from CTRL and LPD/IL-1 $\beta$  animals at P4 analyzed by quantitative RT-PCR. Data (mean  $\pm$  SEM) are relative to Grm3 expression in CTRL animals. Unpaired t-test; ## $p < 0.01$ . (E) Grm3 gene expression assessed in primary microglia from CTRL and LPD/IL-1 $\beta$  animals at P4 after 48h cell culture. Data (mean  $\pm$  SEM) are relative to Grm3 expression in CTRL animals. Unpaired t-test; #### $p < 0.0001$ . (F) Minimal Grm3 network showing the centrality of Grm3 gene deregulation following LPD/IL-1 $\beta$  exposure, selecting deregulated genes that are connected to more than 10 genes. Betweenness centrality of hub genes is quantified by the circle size, and the number of connections among genes by the color code. The figure shows that Grm3 has a central position in the network. (G) The same Grm3 minimal network as in (F) showing the expression of Grm3 and Grm3-associated genes. The blue color versus the red color intensity is associated with down- and up-regulation, respectively.



mGlu3 receptor activation and microglial reactivity in CTRL and LPD/IL-1 $\beta$  cultured microglia at P4. (A-B) Microglial cells were stained with IBA1 (green) and DAPI (blue) under basal and challenged conditions (IL-1 $\beta$  + IFN $\gamma$ )  $\pm$  LY 379268 (1  $\mu$ M) + Ro 64-5229 (25  $\mu$ M). Four cell-culture wells for each condition were analyzed (mean cell number: 165  $\pm$  7) and the cell area, cell perimeter, and cell circularity were assessed. Representative photomicrographs at 40X magnification are shown (scale bar = 50  $\mu$ m). Data (mean  $\pm$  SEM). Two-way ANOVA followed by the Newman-Keuls multiple comparison test; \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 for the effect of LY379268 + Ro 64-5229;  $\$p$  < 0.05,

$$p < 0.01,$$

$\$p$  < 0.001 for the effect of IL-1 $\beta$  + IFN $\gamma$ ; # $p$  < 0.05, ## $p$  < 0.01, ### $p$  < 0.001, #### $p$  < 0.0001 for the effect of LPD/IL-1 $\beta$ . (C) mRNA expression of pro-inflammatory and (D) anti-inflammatory/immune-regulatory markers under basal and pro-inflammatory conditions  $\pm$  LY 379268 (1  $\mu$ M) + Ro 64-5229 (25  $\mu$ M). Data (mean  $\pm$  SEM) are relative to the gene expression under basal CTRL conditions. Two-way ANOVA followed by the Newman-Keuls multiple comparison; \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 for the effect of LY 379268 + Ro 64-5229; ## $p$  < 0.01, ### $p$  < 0.001, #### $p$  < 0.0001 for the effect of LPD/IL-1 $\beta$ ;  $\$p$  < 0.05,

$$\$p < 0.001;$$

$\$\$p$  < 0.0001 for the effect of IL-1 $\beta$  + IFN $\gamma$ .

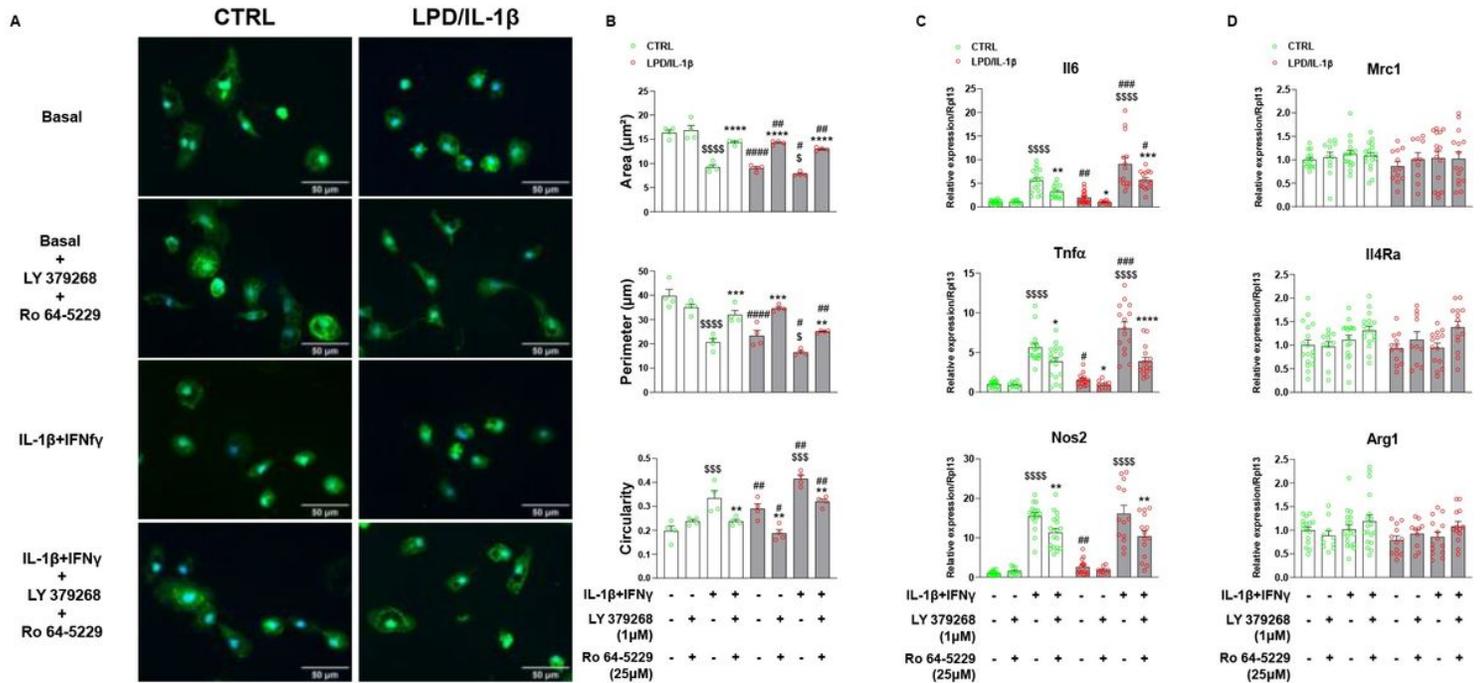


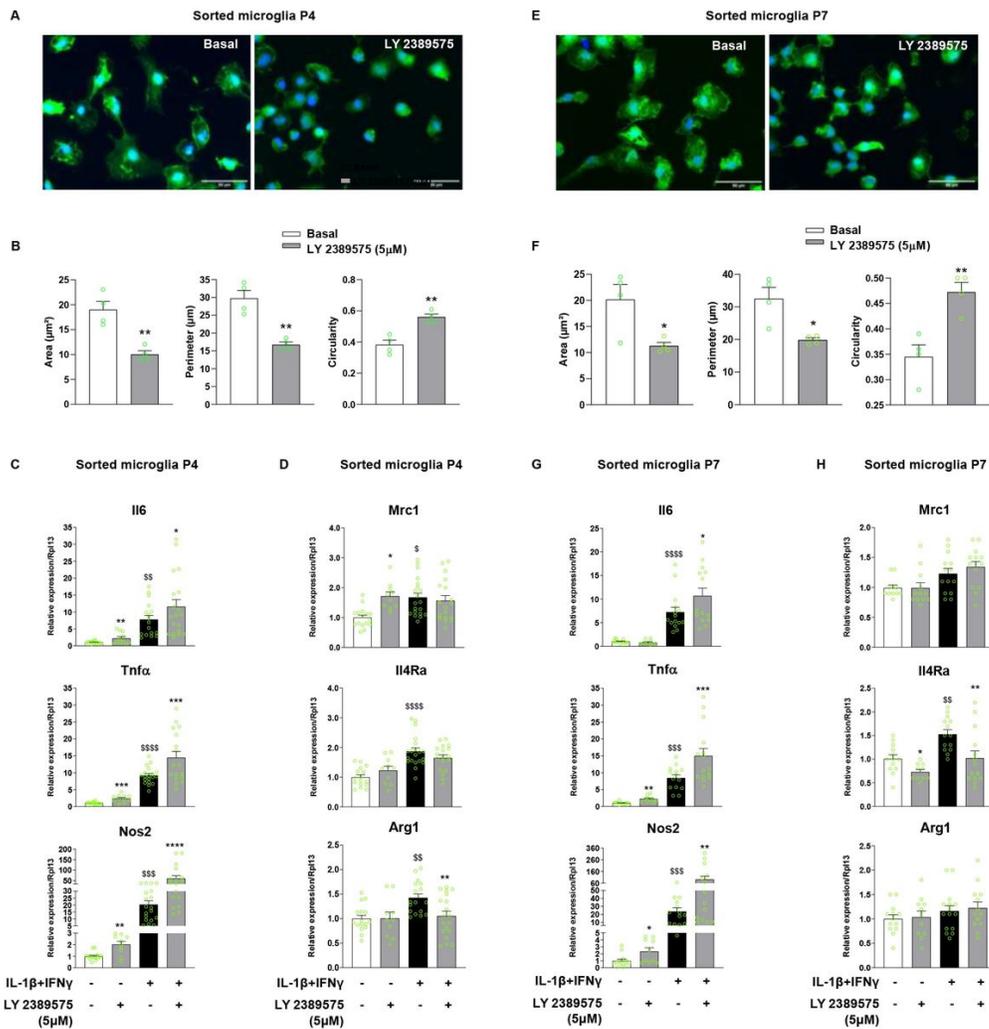
Figure 4

mGlu3 receptor activation and microglial reactivity in CTRL and LPD/IL-1 $\beta$  cultured microglia at P7. (A-B) Microglial cells were stained with IBA1 (green) and DAPI (blue) under basal and challenged conditions (IL-1 $\beta$  + IFN $\gamma$ )  $\pm$  LY 379268 (1  $\mu$ M) + Ro 64-5229 (25  $\mu$ M). Four cell-culture wells for each condition were analyzed (mean cell number: 155  $\pm$  12) and the cell area, cell perimeter, and cell circularity were assessed. Representative photomicrographs at 40X magnification are shown (scale bar = 50  $\mu$ m). Data (mean  $\pm$  SEM). Two-way ANOVA followed by the Newman-Keuls multiple comparison test; \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001 for the effect of LY 379268 + Ro 64-5229;  $\$p$  < 0.05,

$$\$p < 0.001,$$

$p$  < 0.001 for the effect of IL-1 $\beta$  + IFN $\gamma$ ; # $p$  < 0.05, ## $p$  < 0.01, #### $p$  < 0.0001 for the effect of LPD/IL-1 $\beta$ . (C) mRNA expression of pro-inflammatory and (D) anti-inflammatory/immune-regulatory markers under basal and pro-inflammatory conditions  $\pm$  LY

$\$\$p$  < 0.0001 effect of IL-1 $\beta$  + IFN $\gamma$ .



**Figure 5**  
 Pharmacological mGlu3 receptor blockade and microglial reactivity in response to inflammatory stimulation in vitro. (A-B) Microglial cells sorted from P4 pups were stained with IBA1 (green) and DAPI (blue) under basal conditions ± LY 2389575 (5 μM). The cell area, cell perimeter, and cell circularity were assessed (mean cell number: 162 ± 10). Representative photomicrographs at 40X magnification are shown (scale bar = 50 μm). Data (mean ± SEM). Unpaired t-test; \*\*p < 0.01. (C) mRNA expression of pro-inflammatory and (D) anti-inflammatory/immune-regulatory markers in microglial cells sorted from P4 pups cultured under basal or pro-inflammatory (IL-1β + INFγ) conditions ± LY 2389575 (5 μM). The data (mean ± SEM) are relative to the gene expression under basal CTRL conditions. One-way ANOVA followed by the Newman-Keuls multiple comparison test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.001 effect of LY 2389575; §p < 0.05,

$p < 0.01,$

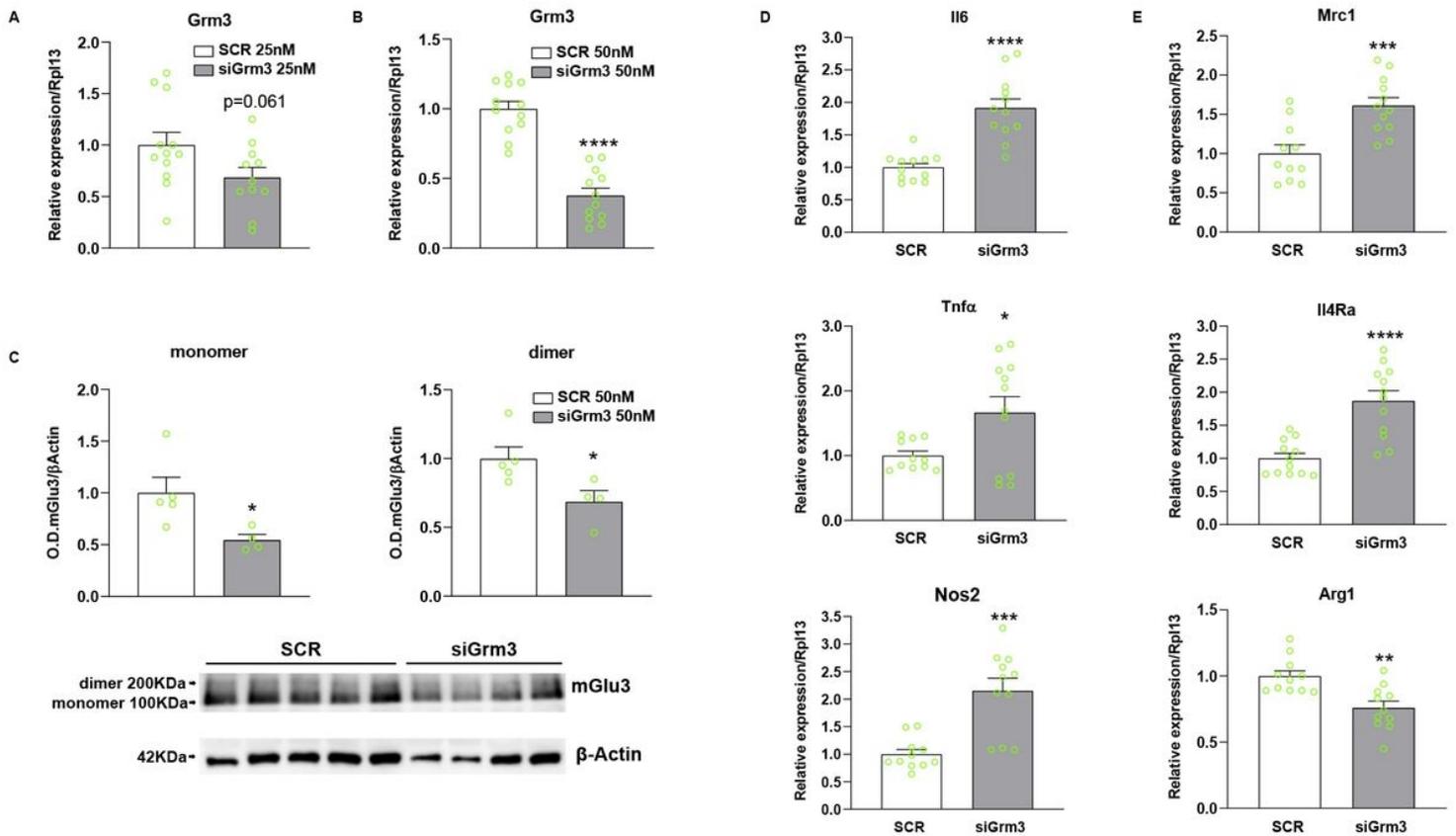
§p < 0.001,

p < 0.0001 effect of IL-1β + INFγ. (E-F) Microglial cells sorted from P7 pups were stained with IBA1 (green) and DAPI (blue) under basal conditions ± LY 2389575 (5 μM). The cell area, cell perimeter, and cell circularity were assessed (mean cell number: 162 ± 10). Representative photomicrographs at 40X magnification are shown (scale bar = 50 μm). Data (mean ± SEM). Unpaired t-test \*p < 0.01, \*\*p < 0.01. (G) mRNA expression of pro-inflammatory and (H) anti-inflammatory/immune-regulatory markers in microglial cells sorted from P7 pups and cultured under basal and pro-inflammatory (IL-1β + INFγ) conditions ± LY 2389575 (5 μM). The data (mean ± SEM) are relative to the gene expression under basal CTRL conditions. One-way ANOVA followed by the Newman-Keuls multiple comparison test \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 effect of LY 2389575;

$p < 0.01,$

§p < 0.001,

p < 0.0001 effect of IL-1β + INFγ.



**Figure 6**

Effect of Grm3 knock-down on microglial reactivity in vitro. (A) Grm3 mRNA expression in primary microglia cultures 48 h after transfection with Grm3 siRNA (25 nM) and (B) with Grm3 siRNA (50 nM). Data (mean ± SEM) are relative to Grm3 expression in the scramble group (SCR). Unpaired t-test; \*\*\*\*p < 0.0001. (C) mGlu3 receptor protein expression in primary microglia cultures 48 h after transfection with Grm3 siRNA (50 nM). Data (mean ± SEM) are relative to mGlu3 receptor protein expression in the scramble group (SCR). Three wells per condition pooled. Unpaired t-test; \*p < 0.05. (D) mRNA expression of pro-inflammatory and (E) anti-inflammatory/immune-regulatory markers in response to SCR and Grm3 siRNA (50nM). Data (mean ± SEM) are relative to the mRNA expression for the respective SCR. Unpaired t-test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

## Supplementary Files

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