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OPPOSITE REGULATION OF METABOTROPIC GLUTAMATE RECEPTOR 3 AND METABOTROPIC GLUTAMATE RECEPTOR 5 BY INFLAMMATORY STIMULI IN CULTURED MICROGLIA AND ASTROCYTES

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Abstract—Metabotropic glutamate receptors (mGluRs) were previously shown to modulate several essential functions in glial cells, including cell proliferation, glutamate uptake, neurotrophic support, and inflammatory responses. As these receptors are regularly proposed as promising targets for the treatment of a wide range of neurological disorders, we herein examined the reciprocal modulation of glial mGluRs by inflammation. Such regulation of mGluRs was also studied in cultures from an experimental model of amyotrophic lateral sclerosis (ALS). Indeed, ALS is characterized by increased neuroinflammation, and glial cell cultures derived from the animal model (rat expressing hSOD1^{G93A}) show enhanced glial reactivity. Within 72 h, the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) induced an increase in mGluR3 and a decrease in mGluR5 gene expression. A similar regulation of these receptors was observed in microglia 48 h after an initial 4-h exposure to lipopolysaccharide. In hSOD1^{G93A}-derived glial cultures, the gene up-regulation of mGluR3 (but not the gene down-regulation of mGluR5) was found to be enhanced in both astrocytes and microglia. Together, these results indicate that an inflammatory environment triggers an opposite regulation in the gene expression of the two predominant mGluR subtypes found in glial cells, and that these regulations were particularly robust in hSOD1^{G93A} glial cultures. As neuroinflammation commonly occurs in several nervous diseases, its influence on mGluR expression should be taken into account when considering these receptors as future drug targets. © 2012 Published by Elsevier Ltd on behalf of IBRO.

Key words: mGluR, astrocyte, microglia, inflammation, ALS, hSOD1^{G93A}.

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Abbreviations: ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; CD11b, cluster of differentiation molecule 11b; COX2, cyclooxygenase 2; GFAP, glial fibrillary acidic protein; hSOD1^{G93A}, mutated human superoxide dismutase 1; IL-1β, interleukin 1β; iNOS, inducible NO synthase; LPS, lipopolysaccharide; mGluR, metabotropic glutamate receptor; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NMDA, *N*-methyl-D-aspartic acid; NO, nitric oxide; ROS, reactive oxygen species; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α.

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Alteration in glutamate handling is a deleterious process implicated in the pathophysiology of several neurological disorders. Indeed, excessive stimulation of glutamate receptors commonly causes excitotoxic insults resulting in neuronal damage/death (Lau and Tymianski, 2010; Sattler and Tymianski, 2001) and activation of surrounding glial cells (Chen et al., 2000; Martinez-Contreras et al., 2002). Neuronal cells express both ionotropic glutamate receptors (ligand-gated ion channels (Kew and Kemp, 2005)), which directly support excitatory transmission, and metabotropic glutamate receptors (mGluRs, G protein-coupled receptors (Ferraguti and Shigemoto, 2006)), which are endowed with modulatory roles (Ozawa et al., 1998). These glutamate targets are also detected in glial cells (D'Antoni et al., 2008), and among the eight known mGluR subtypes, cultured cortical astrocytes were shown to almost exclusively and abundantly express mGluR3 and mGluR5 (Ciccarelli et al., 1997; D'Antoni et al., 2008), while microglia express all mGluRs except mGluR7 (Byrnes et al., 2009b; Pinteaux-Jones et al., 2008; Taylor et al., 2003). The roles of glial mGluRs are not yet fully elucidated but likely involve the regulation of glial activities in response to the local excitatory tone (D'Antoni et al., 2008; Verkhratsky and Kirchhoff, 2007), thereby contributing to glia–neuron interactions. In astroglial cultures, mGluR3 and mGluR5 were respectively shown to negatively and positively influence cell proliferation (Ciccarelli et al., 1997; Kanumilli and Roberts, 2006). These receptors also act as sensors of glutamate homeostasis, through the modulation of the expression (Aronica et al., 2003) or activity (Vermeiren et al., 2005b) of glutamate transporters. Importantly, astrocytes provide neurotrophic support through mGluR-mediated release of growth factors (Bruno et al., 1998; Ciccarelli et al., 1999; Jean et al., 2008). On microglia, mGluRs likely participate in cell migration toward regions showing high glutamate release (Liu et al., 2009a) and modulate the inflammatory phenotype adopted upon activation (Byrnes et al., 2009b; Pinteaux-Jones et al., 2008; Taylor et al., 2003). As mGluR agonists were reported to influence glutamate handling and inflammatory responses in the central nervous system, mGluRs are commonly proposed as relevant targets to treat neurological diseases. Nevertheless, mGluRs are subjected to regulatory processes, and therefore, it appears important to characterize the influence of an inflammatory environment on the expression of mGluRs in glial cells. Making this question even more relevant are those reports showing that neurological disorders that

commonly involve excitotoxicity are also associated with uncontrolled exacerbated neuroinflammation (Minghetti, 2005).

The present study aimed at examining the mGluR3 and mGluR5 gene expression in primary cultures of microglia and astrocytes exposed to defined inflammatory stimuli. These experiments were also conducted in cell cultures obtained from an animal model of amyotrophic lateral sclerosis (ALS, transgenic rats carrying multiple copies of the mutated form of the human superoxide dismutase 1 ($hSOD1^{G93A}$)). Enhanced reactivity to activating stimuli has indeed been already reported in glial cells derived from this transgenic strain (Hensley et al., 2006), which therefore constitutes a relevant model to study the impact of neuroinflammation on mGluR expression. Considering the difficulty to detect mGluR3 at the protein level in cultured glial cells (Aronica et al., 2003; Ciccarelli et al., 1997), we herein focused on mRNA quantifications in glia from both genotypes exposed to neuroinflammatory cues. We moreover examined the expression of specific glial markers, glial fibrillary acidic protein (GFAP) for astrocytes and cluster of differentiation molecule 11b (CD11b) for microglia in the different experimental conditions through mRNA measurements and immunolabeling of glial cultures.

EXPERIMENTAL PROCEDURES

Materials

Poly-L-lysine, tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), lipopolysaccharide (LPS), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (Bornem, Belgium). Culture media, fetal bovine serum, penicillin-streptomycin, fungizone, proline, trypsin-EDTA, and PCR primers were obtained from Invitrogen (Merelbeke, Belgium). TriPure RNA Isolation Reagent was from Roche Diagnostic (Vilvoorde, Belgium). BioRad laboratories (Nazareth, Belgium) provided the iScript cDNA synthesis kit as well as the IQ™ SYBR® Green supermix. Primary antibody rabbit anti-GFAP was from DAKO (Biognost, Heule, Belgium), while primary antibody mouse anti-CD11b was from AbD Serotec (Oxford, UK). The secondary antibody fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG and the Cy3-conjugated goat anti-mouse IgG were obtained from Jackson Immunoresearch Laboratory (DePinte, Belgium). Rabbit anti-mGluR5 was from Upstate (Biognost, Heule, Belgium), while rabbit anti-GAPDH and the peroxidase-conjugated goat anti-rabbit were from Sigma. The chemiluminescence reagents for Western blot were purchased from PerkinElmer (Wellesley, USA). The Percoll™/Redi-Grad™ reagent was purchased from GE Healthcare (Uppsala, Sweden). Culture plasticware were obtained from Greiner Bio-one (Wemmel, Belgium).

Wild-type and $hSOD1^{G93A}$ rats

All experiments were strictly performed respecting the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC). Transgenic Sprague–Dawley rats expressing $hSOD1^{G93A}$ were kindly provided by Dr. R. Pochet (Université Libre de Bruxelles, Belgium). Animals were kept in controlled conditions (temperature, relative humidity, 12-h light/dark cycle) with constant access to food and water. To identify transgenic pups, genomic DNA was extracted from tail biopsies of newborn rat pups (postnatal day 1), and the presence of the transgene was probed by PCR as previously described (Vermeiren et al., 2006).

Astroglial culture

After genotyping, primary cultures from either wild-type or transgenic rat pups (postnatal day 2) were derived from cerebral cortices as previously described (Vermeiren et al., 2005a) with few modifications. Briefly, tissues were isolated and mechanically dissociated in Dulbecco's modified Eagle medium (DMEM) containing glutaMAX, supplemented with fetal bovine serum (10%), proline (50 mg/ml), penicillin-streptomycin (50 mg/ml), and fungizone (2.5 mg/ml). After centrifugation at 1200 rpm during 5 min, cells were resuspended in culture medium, and residual tissue aggregates were eliminated by filtration through a cell strainer (70 μm of pore size) and finally seeded into ventilated culture flasks. Mixed glial cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. On day 7, the medium was renewed, and on day 10, mixed primary cultures reached confluence, allowing astroglial purification by eliminating cell debris, microglia, and oligodendrocytes. To this end, culture flasks were vigorously shaken (orbital shaker, 200 rpm) for a total of 24 h. Two days later, astroglial cultures (>98% positive GFAP-immunoreactive cells) were trypsinized (0.25% trypsin and 1 mM EDTA) and plated at a density of 2.5×10^4 cells/cm^2 in multi-well culture plates. After adherence (24 h), cell maturation was triggered by decreasing the serum concentration to 3%. Culture medium was renewed 4 days later, when astroglial treatment was initiated for 72 h.

Microglial culture

After genotyping, primary cultures from either wild-type or transgenic rat pups (postnatal day 2) were performed from cerebral cortices. Briefly, after isolation, tissues were dissected on ice in 2 ml of cold phosphate buffered saline (PBS, 4 °C) supplemented with 0.324% of glucose and 1% of penicillin-streptomycin. Tissues were then finely minced with scissors and transferred into a culture tube for mechanical dissociation. Culture medium (mix 1:1 of DMEM and F12, supplemented with 1% penicillin-streptomycin) was added to reach a total volume of 15 ml. After 1 min of sedimentation, the supernatant (containing the individualized cells) was transferred into a new tube, and the tissue pellet was again mechanically dissociated in a volume of 20 ml. After 2 min of sedimentation, the supernatant was collected and pooled with the first fraction. The same procedure was performed a third time, and after 3 min of sedimentation, a third fraction of supernatant was collected and pooled. At the bottom of the tube, 1 ml of serum was added before centrifuging at 1000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 35 ml of fresh medium. After adding 1 ml of serum at the bottom, the tube was similarly centrifuged. This step was repeated a third time and cells were finally seeded into 75 cm^2 poly-L-lysine-coated flasks (filtered caps). The culture medium was renewed every 5 days. After 14 days of proliferation, mixed glial cultures were trypsinized to ultimately separate microglia from the other cell types. To this end, stock Percoll was diluted with sterile 10× PBS (9 volumes of stock Percoll and 1 volume of 10× PBS) to yield 100% isotonic Percoll. This 100% isotonic Percoll was then diluted with 1× PBS to obtain 70% and 45% isotonic Percoll. The mixed cell pellet was resuspended in 1 ml of 70% isotonic Percoll and transferred into a sterile 5 ml polystyrene tube. Two ml of 45% isotonic Percoll were then gently layered on top of the 70% layer and finally, 1 ml of PBS was gently layered on top of the 45% Percoll layer. The density gradients were centrifuged at 1200 rpm for 45 min (minimum brake) to obtain two distinct cell layers. After discarding the upper one, the lower layer (between 70% and 45% isotonic Percoll) containing highly enriched microglial cells was gently collected and transferred into an eppendorf. PBS was added to wash the remaining Percoll, and the tubes were centrifuged for 5 min at 3000 rpm. The cell pellet was then resuspended in 1 ml of culture medium, and microglial cells were counted and plated at a density of 5×10^4 cells/cm^2 in multi-well culture plates

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in microglial medium supplemented with 10% FBS. For immunocytochemistry (ICC) experiments (see the ICC section later in the text), the culture plates and cover-slips were previously coated with poly-L-lysine.

Microglial activation

One day after plating, culture medium was replaced by fresh medium containing 10% FBS. The day after, microglial activation was started by replacing the culture medium by medium containing 200 ng/mL of LPS, a potent immune system stimulant considered as the gold standard for inducing microglial activation (Hanisch and Kettenmann, 2007). After 4 h, culture media were replaced in each conditions, and fresh culture medium, containing only 3% of serum, was used. Exactly 48 h after microglial activation was initiated, microglial cultures were terminated, either by adding TriPure Isolation Reagent in each condition (samples were kept at −20 °C until further use) or by fixing cells with ethanol.

Astroglial treatment

Four days after initiating astroglial maturation, the medium was replaced by fresh medium (control condition), or by fresh medium containing TNFα (2, 20, or 50 ng/ml) or IL-1β (1, 10, or 50 ng/ml) (Aronica et al., 2005). The aim of using different concentrations of the stimulus was to potentially highlight an exacerbated sensitivity of hSOD1^{G93A} astrocytes exposed to exactly the same inflammatory environment than wild-type astrocytes. Astroglial treatments always lasted for 72 h and were terminated by adding TriPure Isolation Reagent (samples were kept at −20 °C until further use) or by fixing cells with ethanol.

mRNA extraction, reverse transcription, and real-time PCR measurements

After thawing of samples, RNA extraction was performed according to the manufacturer's protocol. Reverse transcription was carried out with 1 μg of RNA using the iScript cDNA synthesis kit (BioRad, Nazareth, Belgium), in a total volume of 20 μl. Real-time qPCRs were finally performed to specifically amplify CD11b, GFAP, mGluR3, mGluR5, and GAPDH. Reactions were carried out in a total volume of 25 μl, containing 2 ng of cDNA sample, 350 nM of both specific forward and reverse primers previously validated (see Table 1), and the IQ SYBR® Green supermix 1x (BioRad, 100 mM KCl, 40 mM Tris–HCl pH 8.4, 0.4 mM each dNTP, 50 U/ml of iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers). The validated protocol consisted of 45 amplification cycles, each characterized by 15 s of denaturation at 95 °C, 45 s of hybridization at 60 °C, and 15 s of elongation at 79 °C. The measurements were performed using the iCycler IQ multicolor real-time PCR detection system. For quantification, a relative standard curve was generated for each targeted gene by using a cDNA template mix (combining all experimental samples) used at serial dilutions. Relative standard curves were generated by plotting the threshold value (Ct) vs. the log of the amount of total cDNA added to the reaction and used to compare the relative amount of target genes in control and in experimental groups. Signal from each sample was normalized with the relative expression of GAPDH. Calculation of Ct, generation of standard curve, and quantification of mRNA in the samples were performed by the "post run data analysis" software provided with the iCycler system (Bio-Rad Laboratories, Nazareth, Belgium).

Table 1. Real-time qPCR primers (F : forward primer, R : reverse primer) and size of amplicon

Gene	Sequence	Amplicon (bp)
GAPDH	F : 5′- GTCTCCTGTGACTTCAACAG -3′ R : 5′- AGTTGTCATTGAGAGCAATGC -3′	76
GFAP	F : 5′- TGGCCACCAGTAACATGCAA -3′ R : 5′- CAGTTGGCGGCGATAGTCAT -3′	134
CD11b	F : 5′- CTGCCTCAGGGATCCGTAAAG -3′ R : 5′- CCTCTGCCTCAGGAATGACATC -3′	150
mGluR5	F : 5′- AGCTCAACTCCATGATGTTGT -3′ R : 5′- ATCTCTGCGAAGGTCGTCAT -3′	124
mGluR3	F : 5′- CTGGTGATCCTATGCACTGT -3′ R : 5′- GAGGAATGCCAACCAGATGA -3′	120

Immunocytochemistry (ICC)

Cells were grown on coated 12 mm round glass coverslips and underwent the culture and drug treatments. At the end of the treatments, cells were fixed with ethanol (95% v/v) for 15 min at 25 °C. After three washing steps with PBS, cell membranes were permeabilized with Triton X100 (1% v/v in PBS) for 15 min at 25 °C. To prevent non-specific immunostaining, cells were incubated 1 h at 37 °C with a PBS solution containing 3% (w/v) of non fat dry milk. Primary antibody (astrocytes: rabbit anti-rat GFAP (DAKO 1:1000); microglia: mouse anti-rat CD11b (AbD Serotec 1:500)) diluted in a solution 3% milk-PBS was thereafter applied for an overnight incubation at 4 °C. The next day, after three washing steps with a 0.3% milk-PBS solution, cells were incubated in the secondary antibody (FITC-conjugated donkey anti-rabbit or Cy3-conjugated goat anti-mouse (Jackson Immunoresearch 1:500)) diluted in a 3% milk-PBS solution for 1h30 at 25 °C. After three washing steps, nuclei were stained by incubation with the nuclear dye DAPI (1:5000 in PBS) for 15 min at 25 °C. After three rinses, preparations were mounted in Fluoprep (BioMerieux, Brussels, Belgium) and examined using a fluorescent inverted microscope (Evos *fl*, AMG, Westburg, Leusden, Belgium). All the photo-micrographs were taken with the same parameters for the different conditions.

Western blotting

Astrocytes grown in six-well plates were rinsed and scrapped in PBS. After centrifugation at 14,000 rpm for 5 min, proteins were solubilized in the solubilization buffer (10 mM Tris–HCl pH 7.4, 20 mM CHAPS, 0.5 mM EDTA, 30 mM DTT, 0.5 mM PMSF, 1 μM protease inhibitor cocktail). After measuring the protein concentration, each sample was diluted in the loading buffer (125 mM Tris–HCl pH 7.4, 50 mM dithiothreitol, 4%sodium dodecylsulfate, 20%glycerol, 0.01%bromophenol blue, pH 6.8) and stored at −20 °C. For immunoblotting analysis, total protein extracts were boiled for 5 min, electrophoresed through a 7.5% sodium dodecylsulfate-polyacrylamide gel, and transferred to nitrocellulose membranes by electroblotting. To avoid non-specific immunodetection, the membranes were incubated for 1 h in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 5% non-fat milk. Immunoprobing was performed using affinity-purified antibodies recognizing mGluR5 (1/1,200) and GAPDH (1/30,000). Antigen–antibody complexes were detected with a horseradish peroxydase-conjugated goat anti-rabbit IgG secondary antibody (1/3,000). The immunoreactive proteins were detected with enhanced chemiluminescence reagents followed by autoradiography. For quantification, autoradiograms were analyzed by densitometry using the software ImageJ.

Statistical analyses

Data were expressed as arithmetic means with standard error of the mean (SEM), and statistical analysis was performed with GraphPad Prism version 3.02 (GraphPad software, CA, USA). To evaluate if inflammatory treatments induced statistical gene regulation of mGluRs as compared with the respective control group,

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a one-way ANOVA followed by a Dunnett post hoc test were performed in each genotypic group. To compare the different conditions after inflammatory treatments, the Tukey's post-hoc test for multiple comparisons was used, allowing to compare the level of gene expression between both genotypes in control and treated conditions. Values of $P<0.05$ were considered as statistically significant.

RESULTS

Influence of pro-inflammatory stimuli on GFAP and CD11b gene expression and immunoreactivity

The influence of pro-inflammatory cytokines on the astroglial phenotype was examined by comparing the gene expression of GFAP in control cultures or in cells incubated for 72 h with 20 ng/ml of TNFα or 10 ng/ml of IL-1β (Fig. 1A). Quantitative analyses first indicated that GFAP mRNA levels were similar between astrocytes derived from wild-type and transgenic animals maintained in control conditions. Secondly, TNFα was found to decrease GFAP transcript levels in both genotypes, and the amplitude of this effect was similar. IL-1β on the other hand induced a moderate but not significant decrease in GFAP gene expression in wild-type astrocytes, whereas in hSOD1^{G93A} astrocytes, the effect was stronger and significant. When comparing both genotypes, no statistical difference was evidenced for the degree of GFAP gene down-regulation triggered by IL-1β treatment. Astrocytes were also immunostained for GFAP to qualitatively evaluate cell morphology and GFAP-positive signal (Fig. 1C). In control condition, wild-type and hSOD1^{G93A} derived astrocytes showed a typical protoplasmic morphology and a similar GFAP staining. Astrocytes derived from the hSOD1^{G93A} model seemed, however, more stretched and thin, as already reported for astrocytes cultured from the hSOD1^{G93A} mouse model (Hensley et al., 2006). In wild-type astrocytes, exposure to TNFα decreased GFAP immunoreactivity, an effect that was less obvious with IL-1β treatment. Regarding the global morphology of wild-type astrocytes, no clear modification could be noticed following incubations with TNFα or IL-1β. At variance, the intensity of GFAP immunostaining was not modified after incubation with both cytokines in hSOD1^{G93A} astrocytes, some of which, however, appeared hypertrophied in response to the treatments, particularly after TNFα exposure.

Gene expression of the specific microglial marker CD11b was measured in microglial cultures maintained in control conditions or activated with LPS (Fig. 1B). Basal mRNA levels of CD11b were found to be similar in wild-type and hSOD1^{G93A} microglia. To trigger microglial activation and generate an inflammatory environment, microglia were exposed to LPS (200 ng/ml) for 4 h, and CD11b gene expression was examined after a total of 48 h (44 h washout). A significant up-regulation of CD11b gene expression was evidenced in microglial cultures from both genotypes, but this response appeared markedly and significantly enhanced in hSOD1^{G93A} cells. Microglia were immunolabeled for CD11b to qualitatively highlight putative changes in cell morphology (Fig. 2D). In control conditions, microglia appeared as small, round cells, with few cytoplasm and a faint CD11b staining. After LPS activation, increased CD11b immunoreactivity was noticeable in cells from both genotypes, even though the CD11b-positive signal appeared more robust in the hSOD1^{G93A} group. Microglia from both cultures became hypertrophied after activation with LPS.

Regulation of mGluR5 and mGluR3 expression by TNFα and IL-1β in wild-type and hSOD1^{G93A} astrocytes

The influence of inflammation on the expression of mGluR5 in astrocytes was examined in Western blotting. To test the influence of TNFα or IL-1β, these cytokines were added to the cultured medium for 72 h at three different concentrations. As shown in Fig. 2, Western blot analysis performed on samples from wild-type astrocytes revealed that both TNFα and IL-1β considerably and significantly down-regulated mGluR5 immunoreactivity in a concentration-dependent manner. TNFα was more efficient than IL-1β, as exposure to 50 ng/ml for 72 h resulted in 90% and 65% decrease in mGluR5 expression, respectively. Real-time RT-qPCRs were performed on cultured astrocytes to compare the influence of these cytokines on cultured astrocytes from wild-type or hSOD1^{G93A} animals. In control conditions, hSOD1^{G93A} cells showed higher mGluR5 mRNA levels as compared with cells from wild-type littermates (Fig. 3A, B). Challenging TNFα or IL-1β for 72 h at three different concentrations, allowed to detect a putatively higher sensitivity of hSOD1^{G93A} astrocytes. Exposure to TNFα (2, 20, 50 ng/ml) resulted in a substantial decrease in mGluR5 gene expression in astrocytes from both genotypes (up to 90% decrease as compared with corresponding controls). Taking into account the higher expression of the receptor in hSOD1^{G93A} cells in control condition, the relative mGluR5 mRNA down-regulation was similar in astrocytes from the two genotypes (Fig. 3A). Even though the amplitude of the effect was smaller, the levels of mGluR5 mRNA were also decreased by IL-1β in astroglial cultures from both genotypes (Fig. 3B). Thus, the lowest IL-1β concentration tested (1 ng/ml) only caused a modest gene down-regulation of the receptor, whereas a 60% decrease was observed with 50 ng/ml of IL-1β. The amplitude of mGluR5 gene down-regulation induced by IL-1β was similar between wild-type and hSOD1^{G93A} astrocytes.

When studying mGluR3, its basal expression was found to be similar in wild-type and hSOD1^{G93A} astrocytes (Fig. 3C, D). Contrasting with the down-regulation observed for mGluR5, the inflammatory environment triggered a marked mRNA up-regulation of mGluR3. Thus, astrocytes cultured from wild-type animals and exposed to TNFα showed an increase in the gene expression of this receptor, but mGluR3 gene up-regulation was found to be significant only for the TNFα concentration of 50 ng/ml (Fig. 3C). In astrocytes derived from hSOD1^{G93A} rats, the mRNA up-regulation of mGluR3 induced by TNFα was even more intense, reaching statistical difference for the three tested TNFα concentrations. Overall, the increase in mGluR3 gene expression evidenced in hSOD1^{G93A} astro-

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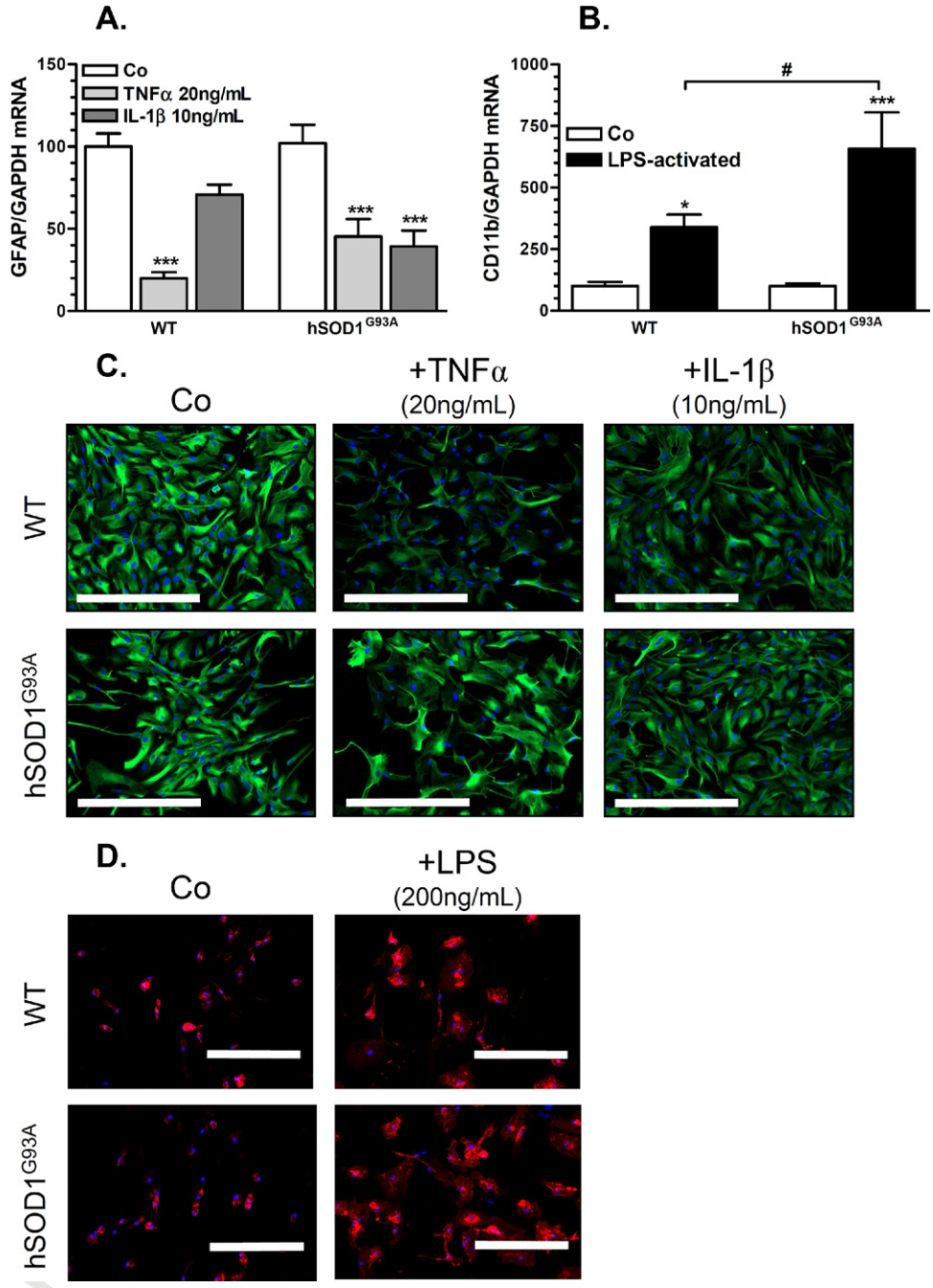

Fig. 1. Influence of pro-inflammatory stimuli on GFAP and CD11b gene expression and immunoreactivity. Gene expression of GFAP (A) and CD11b (B) (normalized for GAPDH gene expression) was examined by RT-qPCR in wild-type and hSOD1^{G93A} cultures of astrocytes and microglia, respectively. Astrocytes were maintained in control conditions or were exposed to TNFα (20 ng/ml) or IL-1β (10 ng/ml) for 72 h. Microglia were left non-activated or were stimulated by LPS (200 ng/ml, 4 h of exposure followed by a washout of 44 h). Shown are mean with SEM from three independent experiments. Statistical analyses were performed through a one-way ANOVA followed by the Dunnett (within each genotypic group, comparisons with the respective control condition) and the Tukey (comparisons of the gene expression level between the two genotypes) post hoc tests (* $P<0.05$ and *** $P<0.001$ for comparisons with respective control; # $P<0.05$ for comparison between genotypes). (C) Representative GFAP immunostaining of wild-type and hSOD1^{G93A} astrocytes in control conditions or after 72 h of treatment with TNFα (20 ng/ml) or IL-1β (10 ng/ml) (scale bar, 400 μm). (D) Representative CD11b immunostaining in wild-type and hSOD1^{G93A} microglia in control or LPS-activated conditions (scale bar, 200 μm). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

cytes after incubation with 20 and 50 ng/ml of TNFα was significantly enhanced as compared with wild-type astrocytes incubated in the same conditions. The same experiments were reproduced using IL-1β (Fig. 3D), which also promoted the higher gene expression of mGluR3, even though statistical significance was only observed with the highest IL-1β concentration (50 ng/ml) tested on wild-type astrocytes. Regarding astroglial cultures obtained from

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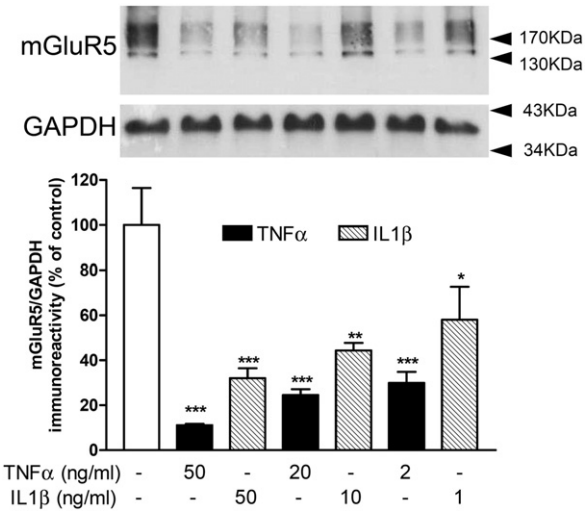

Fig. 2. Influence of TNFα and IL-1β on the expression of mGluR5 in wild-type astrocytes. The relative expression of mGluR5 normalized to GAPDH was examined by Western blotting using specific antibodies. Data in the lower panel indicate the results of densitometric analysis of the immunoreactive signals detected in Western blotting normalized to GAPDH. The upper panel shows a representative experiment. Data shown are mean with SEM from four different experiments. Statistical analyses were performed through a one-way ANOVA followed by the Dunnett post hoc test (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ relative to the control).

transgenic rat pups, both 10 and 50 ng/ml of IL-1β triggered an increase in mGluR3 gene expression. The amplitude of mGluR3 gene up-regulation induced by 50 ng/ml of IL-1β was moreover found to be more intense in hSOD1^{G93A} astrocytes. It is noteworthy that the maximal up-regulations of mGluR3 gene expression obtained with TNFα or IL-1β were similar.

Regulation of mGluR5 and mGluR3 gene expression in wild-type and hSOD1^{G93A} microglia upon exposure to LPS

Constitutive expression of mGluR5 was evidenced in cultured microglia, and mRNA levels of this receptor in control culture conditions appeared twice higher in microglia from the hSOD1^{G93A} model as compared with cells from wild-type littermates (Fig. 4A). To trigger microglial activation, cultures were exposed to 200 ng/ml LPS for 4 h, and mGluR5 gene expression was examined after a total of 48 h. LPS-activated microglia showed a substantial decrease in the expression of mGluR5 in both genotypes (Fig. 4A). The amplitude of gene down-regulation (80%) triggered by the brief exposure to LPS was similar in wild-type and hSOD1^{G93A} microglia.

Gene expression of mGluR3 was also evidenced in cultured microglia but here, no difference in transcript levels could be detected between wild-type and hSOD1^{G93A} microglia in basal conditions (Fig. 4B). After cell activation by LPS, mGluR3 mRNA levels were found to be up-regulated in cultures from both genotypes, but the amplitude of the effect was significantly heightened in hSOD1^{G93A} microglia. Thus, a threefold increased gene expression was measured in wild-type microglia, whereas a sixfold increase was observed for microglia cultured from transgenic rat pups (Fig. 4B).

DISCUSSION

Beside members of the family of glutamate transporters/exchanger and glutamate metabolizing enzymes, glial cells express a variety of mGluRs, which were shown to modulate several activities, including cell proliferation (Ciccarelli et al., 1997; Kanumilli and Roberts, 2006), glutamate uptake (Aronica et al., 2003; Vermeiren et al., 2005b), neurotrophic support (Bruno et al., 1998; Ciccarelli et al., 1999; Viwatpinyo and Chongthammakun, 2009), and inflammatory responses (Byrnes et al., 2009b; Loane et al., 2009). Focusing here on mGluR5 and mGluR3, the two major representatives of group I and II mGluRs, but also the predominant and almost exclusive subtypes of mGluRs expressed in cortical astrocytes (Aronica et al., 2003; Byrnes et al., 2009a; Ciccarelli et al., 1997), we demonstrated that the pro-inflammatory cytokines, TNFα and IL-1β, induced a massive decrease in mGluR5 and an increase in mGluR3 gene expression in cultured astrocytes. The decreased expression of mGluR5 mRNA in response to these cytokines was also demonstrated at the level of the corresponding protein by immunoblotting studies. This could, however, not be confirmed for mGluR3 because of the lack of efficient commercially available antibody. The opposite regulation of mGluR3 and mGluR5 was also observed in microglia upon exposure to LPS. Noteworthy, these two receptors show totally distinct signaling mechanisms. As a member of the $G_{q/11}$-coupled receptor family, mGluR5 is typically identified as a stimulatory receptor, promoting the activation of phospholipase C and downstream responses depending on protein kinase C and Ca^{2+} signals. At variance, mGluR3 is a $G_{i/o}$-coupled receptor inducing the inhibition of adenylyl cyclase and cAMP production. The balanced regulations of these two receptors showing somehow opposite influences on the cellular signaling tone likely constitute an integrated process that contributes to adapt glial cell activities upon inflammatory insults.

ALS is characterized not only by disturbed glutamate handling and excitotoxic damages (Van et al., 2005) but also by exacerbated inflammatory processes (Moisse and Strong, 2006) and oxidative stress (Liu et al., 2007). The question of glial mGluR regulation in response to inflammatory stimuli was therefore extended to this neurodegenerative disorder by culturing astrocytes and microglia from a transgenic rat model of the disease. Showing enhanced responses to inflammatory cues (Hensley et al., 2006), hSOD1^{G93A} glial cultures constitute an interesting model to further characterize the impact of neuroinflammation on selected targets. An increased expression of mGluR3 and mGluR5 was previously evidenced in reactive astrocytes of spinal cord samples of ALS patients (Anneser et al., 2004; Aronica et al., 2001). In the present study, similar levels of mGluR3 mRNA were measured in microglia and astrocytes from newborn wild-type or transgenic rats.

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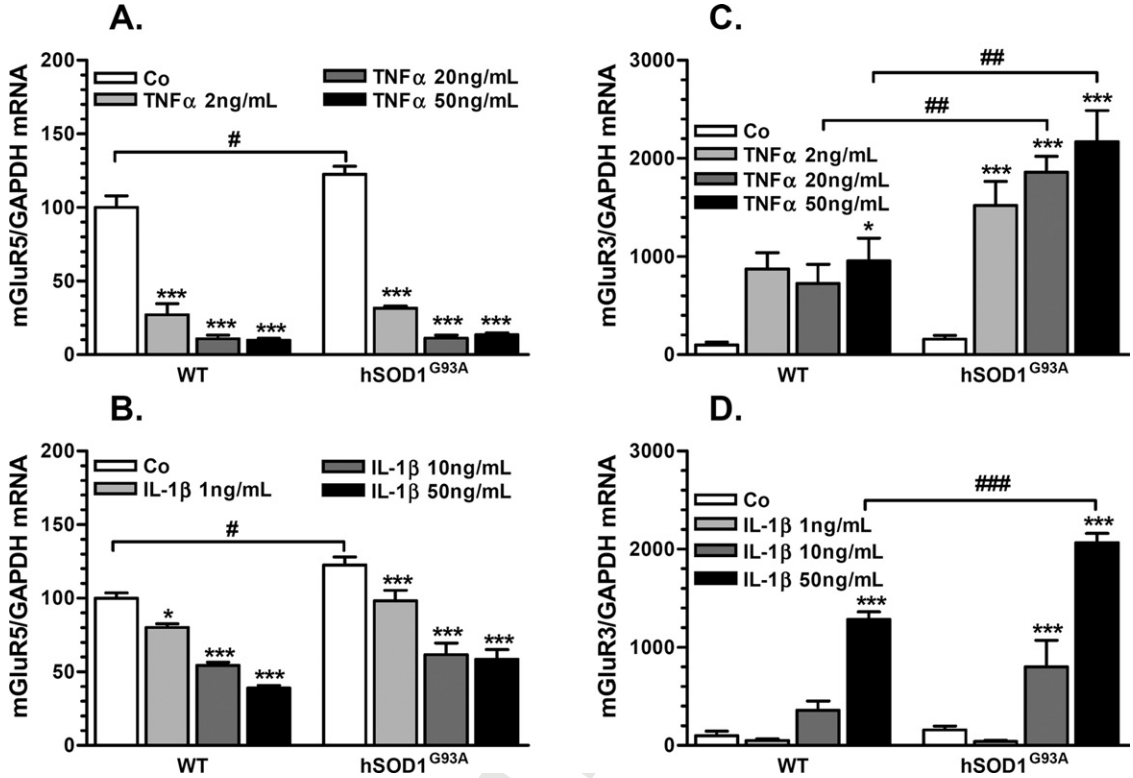

Fig. 3. Influence of TNFα and IL-1β on the expression of mGluR5 and mGluR3 in wild-type and hSOD1^{G93A} astrocytes. Measurements of mGluR5 (A, B) and mGluR3 (C, D) mRNA levels (normalized for GAPDH gene expression) were performed by RT-qPCR in wild-type and hSOD1^{G93A} astrocytes maintained in control conditions or exposed 72 h to TNFα (2, 20, and 50 ng/ml) (panel A, C) or IL-1β (1, 10, and 50 ng/ml) (panel B, D). Shown are mean with SEM from three independent experiments. Statistical analyses were performed through a one-way ANOVA followed by the Dunnett (in each genotypic group, comparisons with the respective control condition) and the Tukey (comparisons of the gene expression level between the two genotypes) post hoc tests (* $P<0.05$ and *** $P<0.001$ for comparisons with respective control; $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$, and $^{\#\#\#}$ $P<0.001$ for comparisons between genotypes).

At variance, mGluR5 gene expression was higher in hSOD1^{G93A} glia as compared with wild-type cells (1.2 and 2 fold higher for astrocytes and microglia, respectively). Accordingly, we previously documented on the higher mGluR5 gene and protein expression in reactive astrocytes derived from hSOD1^{G93A} rats (Vermeiren et al., 2006). The opposite regulation of mGluR3 and mGluR5 triggered by inflammatory cytokines or by LPS in glia from wild-type animals was totally recapitulated in glial cells derived from the hSOD1^{G93A} rats. However, although the amplitude of mGluR5 gene down-regulation was similar between wild-type and hSOD1^{G93A} glial cells, the mRNA up-regulation of mGluR3 was significantly enhanced in both astrocytes and microglia cultured from the ALS model.

Regarding glial markers, the mRNA levels of GFAP (for astrocytes) and CD11b (for microglia) were found to be similar between wild-type and hSOD1^{G93A} cultures maintained in control conditions. Also, the cell morphology was nearly identical between both genotypes, although astrocytes from transgenic rats appeared more thin and elongated, as previously noticed (Hensley et al., 2006). Inflammatory treatments only slightly promoted cell hypertrophy in hSOD1^{G93A} astrocytes without obviously modifying wild-type astrocytes. GFAP mRNA levels were clearly decreased in both wild-type and hSOD1^{G93A} cultures following TNFα treatments, while IL-1β significantly decreased GFAP gene expression only in hSOD1^{G93A} astrocytes. Such GFAP down-regulation was already reported in cultured astrocytes after cytokine exposure (Focant et al., 2011; Krohn et al., 1999; Oh et al., 1993; Selmaj et al., 1991), or after treatments with conditioned media from activated microglia (Rohl et al., 2007; Tilleux et al., 2007). In these studies and corroborating the present results, despite the GFAP gene down-regulation and increased proliferation, no changes were detected in GFAP protein expression (Oh et al., 1993) or on cell morphology (Rohl et al., 2007). Regarding microglia, LPS triggered the gene up-regulation of CD11b in both genotypes. These changes were, however, clearly enhanced in hSOD1^{G93A} microglia, validating the inflammatory-prone properties of the ALS model, as suggested *in vitro* (Xiao et al., 2007) and *in vivo* (Berger et al., 2011).

Down-regulation of mGluR5 was already evidenced in differentiated astrocytes treated with IL-1β (Aronica et al., 2005) or thrombin (Miller et al., 1996), and in naive astrocytes incubated with conditioned media from LPS-activated microglia (Tilleux et al., 2007), both at the mRNA and protein levels. On microglia however, mGluR5 regulation after activation *in vitro* was never characterized so far.

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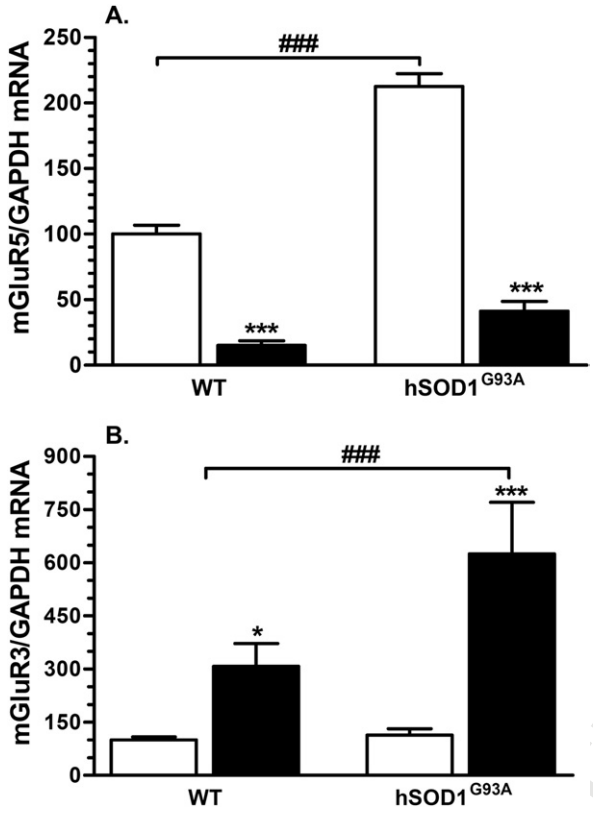

Fig. 4. Influence of LPS activation on the expression of mGluR5 and mGluR3 in wild-type and hSOD1^{G93A} microglia. Measurements of mGluR5 (A) and mGluR3 (B) mRNA levels (normalized for GAPDH gene expression) were performed by RT-qPCR in wild-type and hSOD1^{G93A} microglia maintained in control or in LPS-activated (200 ng/ml, 4 h of exposure followed by a washout of 44 h) conditions. Shown are mean with SEM from three independent experiments. Statistical analyses were performed through a one-way ANOVA followed by the Dunnett (in each genotypic group, comparisons with the respective control condition) and the Tukey (comparisons of the gene expression level between the two genotypes) post hoc tests (* $P<0.05$ and *** $P<0.001$ for comparisons with respective control; ### $P<0.001$ for comparisons between genotypes).

Decreased expression of mGluR5 in inflammatory conditions was rather unexpected as several pathological models were shown to be associated with an mGluR5 up-regulation, such as epilepsy (Aronica et al., 2000), kainate-induced neuronal injury (Ferraguti et al., 2001), multiple sclerosis (Geurts et al., 2003), or excitotoxic ibotenic acid hippocampal lesions (Drouin-Ouellet et al., 2011). At variance with our study, these pathological models take into account the persistent crosstalk existing between the different cell types, leading to an inflammatory environment enriched in variety of mediators originating from multiple sources. Furthermore, while our *in vitro* study focuses on neuroinflammation, it is likely that other cellular mechanisms contribute to the regulation of mGluR5 in these *in vivo* models of nervous disorders.

In astrocytes, stimulation of mGluR5 is known to promote brain-derived neurotrophic factor (BDNF) expression (Jean et al., 2008; Viwatpinyo and Chongthammakun, 2009), which sensitizes motor neurons to glutamate-dependent excitotoxic insults (Hu and Kalb, 2003). Hence, the increased gene expression of mGluR5 in non-treated hSOD1^{G93A} astrocytes may contribute to the vulnerability of motor neurons in ALS. Accordingly, protection against excitotoxicity was achieved through prolonged pharmacological blockade of astroglial mGluR5, via a mechanism involving BDNF modulation and down-regulation of the Ca^{2+}-permeable GluR1 subunit in neurons (D'Antoni et al., 2011). Furthermore, activation of mGluR5 was shown to decrease glutamate transporter expression in astrocytes (Aronica et al., 2003), an effect that would expose neuronal cells to elevated concentrations of extracellular glutamate. From these observations, the decreased expression of mGluR5 in response to inflammation constitutes a protective adaptation against excitotoxicity. Nevertheless, on microglia, stimulation of mGluR5 seems to rather support neuroprotection as it attenuates reactivity and toxicity of LPS-activated microglia through reduction of nitric oxide (NO), reactive oxygen species (ROS), and TNFα production (Byrnes et al., 2009b; Loane et al., 2009). Although our data indicate that the mGluR5 gene regulation of by inflammation is preserved in hSOD1^{G93A} glia, the review of the literature illustrates the complex influence of glial mGluR5 on neuron viability, and it remains difficult to speculate on the specific implication of these receptors in the context of ALS.

AQ: 8

Regarding mGluR3, we evidenced in both astrocytes and microglia a robust up-regulation at the mRNA level, whereas the detection at the protein level is extremely difficult in glial cultures (Aronica et al., 2003; Ciccarelli et al., 1997), even after the strong up-regulation triggered by inflammatory treatments (data not shown). It was reported that activation of this receptor on astrocytes promotes the release of TGFβ, which mediates protection of neurons against NMDA-induced toxicity (Bruno et al., 1998; Corti et al., 2007), whereas on microglia it attenuates the myelin-evoked neurotoxicity (Pinteaux-Jones et al., 2008). Furthermore, activation of mGluR3 on astrocytes was shown to increase glutamate transporter expression (Aronica et al., 2003), ensuring a better control of extracellular concentrations of glutamate. Together, these data indicate that the up-regulation of mGluR3 reflects an overall neuroprotective phenotype adopted by glial cells in response to inflammation. It is tempting to propose that the amplified response evidenced in the hSOD1^{G93A} model endow the glial cells with a heightened protective profile. At the molecular level, this increased mGluR3 gene up-regulation may reflect a higher sensitivity of hSOD1^{G93A} cells to the activating stimulus. It was indeed previously demonstrated that hSOD1^{G93A} astrocytes exposed to TNFα show reinforced NO production, up-regulation of pro-inflammatory genes, and protein carbonylation (Hensley et al., 2006). The enhanced gene up-regulation of mGluR3 in hSOD1^{G93A} astrocytes, however, contrasts with the lack of exacerbated gene down-regulation of mGluR5. As the intracellular effectors leading to the increase in mGluR3 gene expression after inflammatory treatments remain unknown, elucidating the mechanisms that support the amplified effect in an ALS context is so far delicate. Nevertheless, it is documented that

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activation of glia expressing mutated hSOD1: (i) increases NADPH oxidase activity and ROS production (Liu et al., 2009b), (ii) enhances iNOS expression and NO release (Xiao et al., 2007), and (iii) increases COX2 expression (D'Ambrosi et al., 2009). Such mechanisms could then possibly participate in the enhanced regulation observed for mGluR3 mRNA levels without influencing mGluR5 gene down-regulation.

CONCLUSION

In summary, we herein provide evidence for an opposite regulation of the gene expression of the principal members of family I and II mGluRs in glial cells in response to inflammatory cues. These observations raise questions regarding the relevance of targeting these mGluRs in the treatment of neurological diseases. Also, while the increased gene expression of mGluR3 and the opposite silencing of mGluR5 can be considered as constitutive adaptive processes driving the glial cells into neuroprotective phenotypes, these data also raise concerns regarding the consequences of pharmacologically manipulating neuroinflammatory processes. Undoubtedly, further studies are crucial to unravel the importance of these mGluRs in both physiological conditions as well as in neurological disorders that putatively involve neuroinflammation.

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