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RESEARCH ARTICLE

Constitutive downregulation protein kinase C epsilon in hSOD1^{G93A} astrocytes influences mGluR5 signaling and the regulation of glutamate uptake

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Abstract

Accumulating evidence indicates that motor neuron degeneration in amyotrophic lateral sclerosis (ALS) is a non-cell-autonomous process and that impaired glutamate clearance by astrocytes, leading to excitotoxicity, could participate in progression of the disease. In astrocytes derived from an animal model of ALS (hSOD1^{G93A} rats), activation of type 5 metabotropic glutamate receptor (mGluR5) fails to increase glutamate uptake, impeding a putative dynamic neuroprotective mechanism involving astrocytes. Using astrocyte cultures from hSOD1^{G93A} rats, we have demonstrated that the typical Ca^{2+} oscillations associated with mGluR5 activation were reduced, and that the majority of cells responded with a sustained elevation of intracellular Ca^{2+} concentration. Since the expression of protein kinase C epsilon isoform (PKCε) has been found to be considerably reduced in astrocytes from hSOD1^{G93A} rats, the consequences of manipulating its activity and expression on mGluR5 signaling and on the regulation of glutamate uptake have been examined. Increasing PKCε expression was found to restore Ca^{2+} oscillations induced by mGluR5 activation in hSOD1^{G93A}-expressing astrocytes. This was also associated with an increase in glutamate uptake capacity in response to mGluR5 activation. Conversely, reducing PKCε expression in astrocytes from wild-type animals with specific PKCε-shRNAs was found to alter the mGluR5 associated oscillatory signaling profile, and consistently reduced the regulation of the glutamate uptake-mediated by mGluR5 activation. These results suggest that PKCε is required to generate Ca^{2+} oscillations following mGluR5 activation, which support the regulation of astrocytic glutamate uptake. Reduced expression of astrocytic PKCε could impair this neuroprotective process and participate in the progression of ALS.

KEYWORDS
amyotrophic lateral sclerosis, calcium oscillations, metabotropic glutamate receptor, protein kinase C

1 | INTRODUCTION

Apart from its role as the principal excitatory neurotransmitter in the CNS, the amino-acid L-glutamate is also actively involved in the communication between neurons and glia, in particular astrocytes (Santello, Cali, & Bezzi, 2012). Indeed, glutamate interacts with a variety of G-protein coupled receptors (metabotropic glutamate receptors, mGluR) which are detected on synaptic terminals (Yin & Niswender, 2014) as well as on astrocytes (D'Antoni et al., 2008). The best characterized of these metabotropic receptors is the type 5 receptor (mGluR5) which is expressed in cultured astrocytes where it contributes to the regulation of glial responses to changes in local excitatory tone (D'Antoni et al., 2008; Verkhratsky & Kirchhoff, 2007). Through a functional coupling with $G\alpha_{q/11}$ proteins, astrocytic mGluR5 mediates increases in the intracellular Ca^{2+} concentration and triggers the release of gliotransmitters such as ATP, neurotrophic factors, D-serine, glutamate and GABA, which in turn impact on neuronal responses (Bazargani & Attwell, 2016; Jean, Lercher, & Dreyfus, 2008; Panatier et al., 2011; D'Ascenzo et al., 2007).

Several Ca^{2+} signaling profiles have been observed upon mGluR5 activation in astrocytes. Depending on the cell density, the level of

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receptor expression or colocalization with mGluR5 signaling partners, single-peak responses, peak-plateau responses or Ca^{2+} oscillations have been reported (Bradley, Watson, & Challiss, 2009; Nakahara, Okada, & Nakanishi, 1997; Morita et al., 2003; Bradley & Challiss, 2011). In several models, it has been suggested that distinct Ca^{2+} signaling profiles differentially regulate down-stream effectors (Dupont & Combettes, 2016), and the dynamic of Ca^{2+} oscillations has received increasing attention as it could influence the nature or the amplitude of functional cellular responses. Nevertheless, while Ca^{2+} oscillations in astrocytes have frequently been reported in response to a variety of stimuli, their functional significance remains poorly understood. In previous studies, astrocytes derived from a transgenic rats over-expressing a mutated form of superoxide dismutase ($hSOD1^{G93A}$), a commonly used animal model for familial amyotrophic lateral sclerosis (ALS), we have previously correlated an altered mGluR5-mediated Ca^{2+} signaling profile with the impairment of a mGluR5-mediated increase in glutamate uptake (Vermeiren et al., 2006). A critical role for mGluR5 has been demonstrated in several neurological disorders such as the fragile X syndrome (Dolen & Bear, 2008), epilepsy (Ure, Baudry, & Perassolo, 2006), Huntington's disease (Ribeiro et al., 2014), Alzheimer's disease (Hamilton et al., 2016) and ALS (Anneser, Chahli, & Borasio, 2006). However, neither the role of astrocytic mGluR5 nor the alteration in its signaling profile have been investigated in these disorders.

Several intracellular protein partners interact with mGluR5 and influence its intracellular signaling (Fagni, 2012). In particular, a critical role has been assigned to PKC epsilon (PKCε) in the control of mGluR5-mediated Ca^{2+} oscillations, both in transfected cells and in cultured astrocytes (Bradley & Challiss, 2011). Using diverse pharmacological inhibitors or genetic tools, the authors have demonstrated that expression of PKCε and its activity are essential for the maintenance of mGluR5-mediated Ca^{2+} oscillations. We also identified a role for this PKC isoform and the associated Ca^{2+} oscillations in the prevention of mGluR5 desensitization upon repeated activation (Vergouts et al., 2017). Considering the importance of mGluR5 in the control of astrocytic functions, alteration in its signaling through molecular partners, such as PKCε could indirectly contribute to an impairment of neuroprotective support, as observed in ALS. We have therefore analyzed a putative dysregulation of PKCε in $hSOD1^{G93A}$ astrocytes and its implication in mGluR5-associated responses. In the present study we provide evidence for a constitutive downregulation of PKCε in astrocytes derived from $hSOD1^{G93A}$ rodents ($hSOD1^{G93A}$ astrocytes), and show that this alteration is correlated with a loss of Ca^{2+} oscillations upon mGluR5 activation. We also demonstrate that rescuing PKCε expression in $hSOD1^{G93A}$ astrocytes restores mGluR5-mediated Ca^{2+} oscillations and promotes mGluR5-mediated up-regulation of glutamate uptake.

2 | MATERIALS AND METHODS

2.1 | Materials

Poly-L-lysine, the rabbit anti-glyceraldehyde-3-phosphate deshydrogenase (GAPDH) antibody and goat anti-rabbit and goat anti-mouse IgG secondary antibodies were all obtained from Sigma Aldrich (Diegem, Belgium). All cell culture reagents, including the G5 supplement and foetal bovine serum (FBS), were obtained from Thermofisher (Merelbeke, Belgium). (S)-3,5-dihydroxyphenylglycine (DHPG), Ro 31–8220, Gö 6976, and FR 236924 were purchased from Tocris (Bristol, UK) and fura2 acetoxymethyl-ester from Molecular Probes (Asse, Belgium). The rabbit anti-mGluR5 antibody was purchased from Upstate (Biognost, Heule, Belgium). The mouse PKCε antibody was obtained from BD Bioscience (Erembodegem, Belgium). The DC Protein Assay Reagents Package for protein dosage was from BioRad Laboratories (Nazareth, Belgium) and the chemiluminescence reagents for immunoblot experiments were purchased from PerkinElmer NEN (Zaventem, Belgium). The Percoll/RediGrad reagent was purchased from GE Healthcare (Uppsala, Sweden). Culture plasticwares were obtained from Greiner Bio-One (Wemmel, Belgium).

2.2 | Animals and ethics statement

All experiments were conducted on primary cell cultures derived from Sprague-Dawley rat pups in strict adherence to the EU directive of 22/09/2010 (2010/63/EU) and with the agreement of the Belgian Ministry of Agriculture (code number LA 1230297). The ethical committee of the Université catholique de Louvain for animal experiments specifically approved this study (code number 2010/UCL/MD/032). All animals used for breeding were housed in cages in controlled light/dark cycles, temperature and humidity. Every effort was made to minimize any suffering during the manipulations and pups were sacrificed by decapitation.

2.3 | Primary cultures of cortical rat astrocytes

Cortices from rat pups were collected at postnatal day 2 and mechanically dissociated. Astrocytes were separated from other cell types using a 30% Percoll gradient and were seeded into gelatin-coated tissue culture flasks. Cells were left to proliferate for 14 days at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM-glutaMAX) supplemented with 10% FBS, 50 mg/ml penicillin–streptomycin and 50 mg/ml fungizone. On day 7, the medium was renewed, and on day 14 cells were passaged and cultured in DMEM-glutaMAX supplemented with 10% FBS. Two days later, FBS was reduced to 3% and supplemented with the growth factor cocktail G5. All experiments were conducted 7 days later.

2.4 | Construction of lentiviral vectors and cell transduction

Short hairpin RNA (shRNA) sequences targeting rat PKCε (CCCTTATCTAACCCAACTCT and CGTCACTTCGAGGACTGGATT) transcript were selected using RNA mission collection (Sigma). They were cloned in pMK117 (Kreit, Vertommen, Gillet, & Michiels, 2015) using BshTI and EcoRI. This construct also harbors the gene encoding for the fluorescent protein E2-Crimson, facilitating the detection of infected cells. Lentiviral particles were produced by transfection of 293T cells with 15 μg of lentiviral vectors, 11.25 μg of pSPAX2 (Addgene) and 4.5 μg of pMD2-VSV-G. Lentiviral particles in supernatant were collected 24

and 48 h post transfection, passed through a 0.45 μm filter and concentrated 10× using Vivaspin columns (cutoff: 1,000,000 Da; Vivaspin). The resulting lentiviral particles were titrated by flow cytometry and by transducing 10,000 HEK293 cells with serial dilutions of lentiviruses followed by detection of E2-Crimson positive cells.

The coding sequence of PKCε was amplified from total rat brain cDNA by PCR, using Kapa HiFi HotStart polymerase (Kapa Biosystems KK2502), vPKC-F and vPKC-R primers. The amplified fragment was cloned into pTM945 (De Cock & Michiels, 2016), using BshTI and BsiWI restriction enzymes. The cloned PKCε sequence was validated by double strand sequencing to ensure the absence of mutation introduced during the PCR amplification step. Lentiviral particles were generated by transient transfection of 293T cells grown in 75-cm^2 dishes. 15 μg of lentiviral vectors (pTM945 and PKCε), 7.5 μg of pMDLg/RRE (Gag-Pol), 4.5 μg of pMD2-VSV-G (VSV-glycoprotein) and 3.75 μg of pRSV-Rev (Rev) were transfected. Cell supernatants were harvested 24 and 48 hr post transfection and passed through a 0.45 μm filter.

2.5 | Single-cell monitoring of intracellular [Ca^{2+}] variations

Changes in [Ca^{2+}]$_i$ were measured in single astrocytes using the Ca2+ sensitive fluorescent dye Fura2. Astrocytes grown on poly-L-lysine-coated 15 mm round glass coverslips were loaded with 1 μM Fura2-acetoxymethyl ester for 40 min in Krebs buffer (118 mM NaCl, 4.7 mM KCl, 4 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 8.5 mM Hepes pH 7.4, 1.3 mM $CaCl_2$, 11.7 mM glucose). Coverslips were rinsed once and then mounted in a heated (37°C) and perfused microscope chamber (Warner Instrument Corporation, Hamden, CT). Loaded astrocytes were excited successively (2 Hz) at 340 and 380 nm (excitation light was obtained from a xenon lamp coupled to a monochromator) for 100 ms and emitted fluorescence was monitored at 510 nm using a charged coupled device sensor (CDD) camera coupled to an inverted Olympus IX70 microscope (TILL photonics, Martinsied, Germany). Fluorescence intensities from single astrocytes, excited at the two wavelengths, were recorded separately, corrected for the background and combined (fluorescence ratio F340/F380) using the software TILLvisION version 3.3.

2.6 | Immunoblotting

Astrocytes in 6-well plates were rinsed with PBS and scraped in ice-cold lysis buffer (10 mM TrisHCl pH 7.4, 1 mM EDTA, 10 mM EGTA, 2 mM DL-dithiotreitol (DTT), 1% Igepal-NP40, 20% glycerol). Samples were collected and then centrifuged at 1,000*g* for 3 min to remove insoluble material. Protein concentration was determined using the DC Protein Assay Reagents Package and samples were diluted in a loading buffer (125 mM TrisHCl pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS) and 0.01% bromophenol blue) and boiled for 5 min. Samples were electrophoresed through a 10% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Membranes were incubated 1 h in Tris-buffered saline (TBS – 50 mM TrisHCl pH 7.4, 150 mM NaCl) containing 0.05% Tween-20 and 5% non-fat milk to reduce non-specific labeling. Immunostaining of the blots was carried out by incubating membranes overnight at 4°C with the following primary antibodies: rabbit anti-mGluR5 (1/1200), mouse anti-PKCε (1/1000) and rabbit anti-GAPDH (1/30,000). Membranes were then incubated 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1/3,000) or peroxidase-conjugated goat anti-mouse IgG (1/2,000). Immunoreactive proteins were detected with enhanced chemiluminescence reagent followed by autoradiography. Densitometric analysis of protein signals were performed using ImageJ (NIH).

2.7 | Glutamate uptake

Primary cultured astrocytes were grown on 12-well plates coated with poly-L-lysine. Plates were placed on the surface of a 37°C water bath and were rinsed twice with 1 ml of preheated sodium Krebs buffer (25 mM HEPES pH 7.4, 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM MgSO4, 6 mM glucose). In the glutamate uptake experiments, radiolabeled D-aspartate (D-[^{3}H]-aspartate, specific activity of 11.3 Ci/mmol, Perkin Elmer) was used as substrate as it is a transportable analogue of L-glutamate which is not metabolized and does not interact with glutamate receptors. D-[^{3}H]-aspartate (20 nM) was diluted with unlabeled L-aspartate to achieve a final concentration of 100 μM. When tested, the PKC inhibitors Ro 31–8220 (5 μM) and Gö 6976 (10 μM) and the PKC activator FR 236924 were added 6 min before the uptake assay. Also, when the influence of DHPG was tested, it was added 15 s before addition of the radiolabeled substrate. After 6 min, uptake was stopped by three rinses with cold sodium-free Krebs buffer (25 mM HEPES pH 7.4, 120 mM choline, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM MgSO4, 6 mM glucose) and cells were lysed with 0.1 N NaOH. Radioactivity was measured using the liquid scintillation solution Microscint 40 and the TopCount NXT Microplate Scintillation and Luminescence Counter (Perkin Elmer). A fraction of the lysate was also used for protein determination by the Bradford method using BioRad Protein Assay Dye Reagent (BioRad).

2.8 | Statistical analysis

Data were expressed as means with the standard error of the mean (SEM) and statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, CA). Differences in experimental data among two different groups were evaluated with Student's *t* test. Analysis of variance between multiple groups was conducted through a one-way ANOVA followed by a Dunnett's test for multiple comparisons to compare all different conditions to the control group. In all statistical analyses, a value of $P < .05$ was defined as significant.

3 | RESULTS

3.1 | Altered function of mGluR5 in cultured astrocytes derived from newborn hSOD1^{G93A} rats

The functional properties of mGluR5 in primary cultured astrocytes derived from wild-type (WT) or hSOD1^{G93A} rats were examined after one week of cell maturation in FBS 3% medium supplemented with a

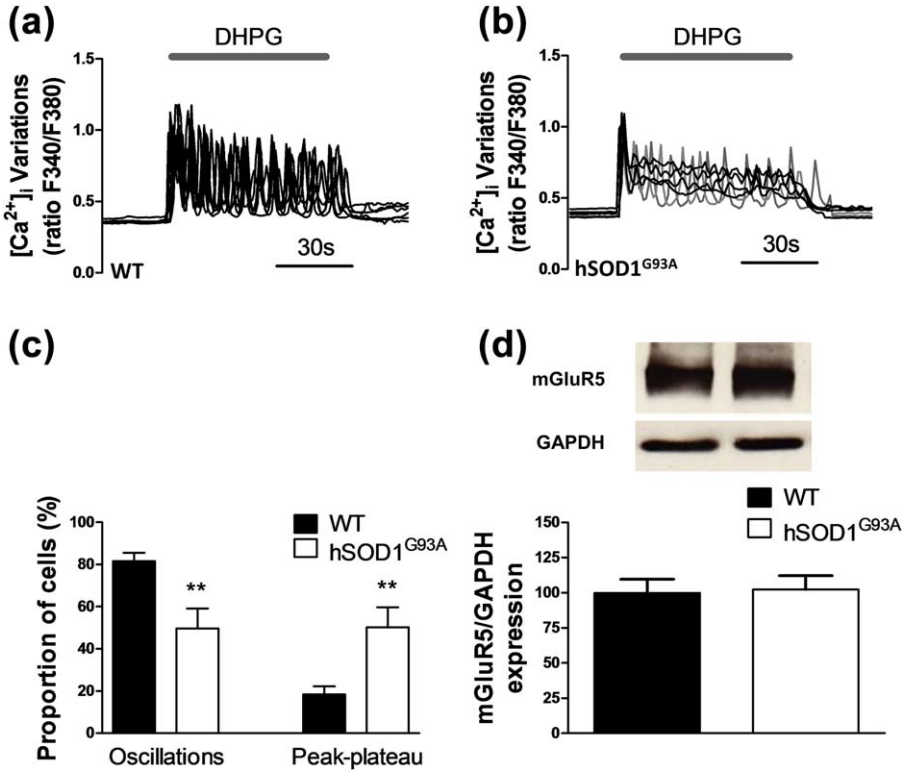

FIGURE 1 mGluR5 signaling and expression in rat cortical astrocyte cultures. Astrocytes were exposed to 50 μM DHPG for 60 s and cytosolic Ca^{2+} mobilization was monitored using Fura2. Representative traces indicating calcium responses in 6 different cells are shown from WT (a) or hSOD1^{G93A} (b) astrocytes. Panel c illustrates the proportion of peak-plateau profiles or calcium oscillations in these cells. Histograms show means with *SEM* of 12 different experiments. Statistical analyses were performed through a *t* test (**$P < .01$). Expression of mGluR5 (d) was examined by immunoblotting. Blots shown are representative of four independent experiments that were all included for densitometric analyses of immunoreactive bands. Histograms show means with *SEM* of four different experiments. After normalization to GAPDH expression, data were shown as relative to the signal obtained from WT astrocytes. Statistical analyses were performed through a *t* test and no significant differences were observed [Color figure can be viewed at wileyonlinelibrary.com]

standardized cocktail of growth factors (G5 supplement). In cultures derived from WT animals, the majority of astrocytes (80%) showed typical Ca^{2+} oscillations upon exposure to 50 μM DHPG (Figure 1a,c). Under the same conditions, <50% of hSOD1^{G93A} astrocytes showed Ca^{2+} oscillations (Figure 1b,c; $P = .0021$), while the rest of the cells responded with an initial peak of similar amplitude as the first oscillation, which was followed by a sustained plateau phase. Both WT and of hSOD1^{G93A} astrocytes showed a rapid extinction of the calcium signals upon DHPG washout. .

The expression of mGluR5 was analyzed on crude cell extracts from both cultures by immunoblotting. As shown in Figure 1d, no significant difference in the mGluR5 expression level was observed between WT or hSOD1^{G93A} astrocytes ($P = .86$). This suggests that the altered signaling profile in astrocytes derived from transgenic animals is not caused by an altered expression of the receptor.

3.2 | PKCε activity and expression influence the mGluR5-mediated Ca^{2+} signaling in astrocytes derived from both WT and hSOD1^{G93A} rats

A direct role of PKCε in the control of mGluR5-mediated Ca^{2+} oscillations has been described and we have recently confirmed that a reduction of expression in WT astrocytes causes a loss of Ca^{2+} oscillations in response to DHPG (Bradley & Challiss, 2011; Vergouts et al., 2017). Therefore, the expression of PKCε was determined in total cell extracts of astrocytes derived from both WT and hSOD1^{G93A} rats. Immunoblot analyses revealed a significant (i.e., five-fold) reduction of PKCε expression in hSOD1^{G93A} astrocytes (Figure 2a; $P = .0071$). The implication of reduced PKCε expression in the altered response to DHPG was examined through pharmacological manipulation of its activity. As previously shown (Vergouts et al., 2017), pre-treatment of WT astrocytes with the pan-PKC inhibitor Ro 31–8220 considerably reduced the proportion of cells showing Ca^{2+} oscillations in response to DHPG exposure and almost all cells showed a peak-plateau type response (Figure 2b,c). In contrast, the oscillatory pattern observed in these astrocytes was not altered when exposed to Gö 6976, which inhibits Ca^{2+}-dependent PKC isoforms and does not act on PKCε (Figure 2d). Similar results were obtained in hSOD1^{G93A} astrocytes as inhibition of all PKC with Ro-31–8220 led to a total loss of the oscillatory-type response to DHPG (data not shown). With the goal of promoting an oscillatory profile in these cells, PKCε was stimulated by exposing astrocytes to the specific activator FR 236924 for 30 min. However, none of the conditions tested (from 0.1 to 100 nM) were effective in increasing the proportion of cells showing oscillations in response to DHPG (Figure 2e–h).

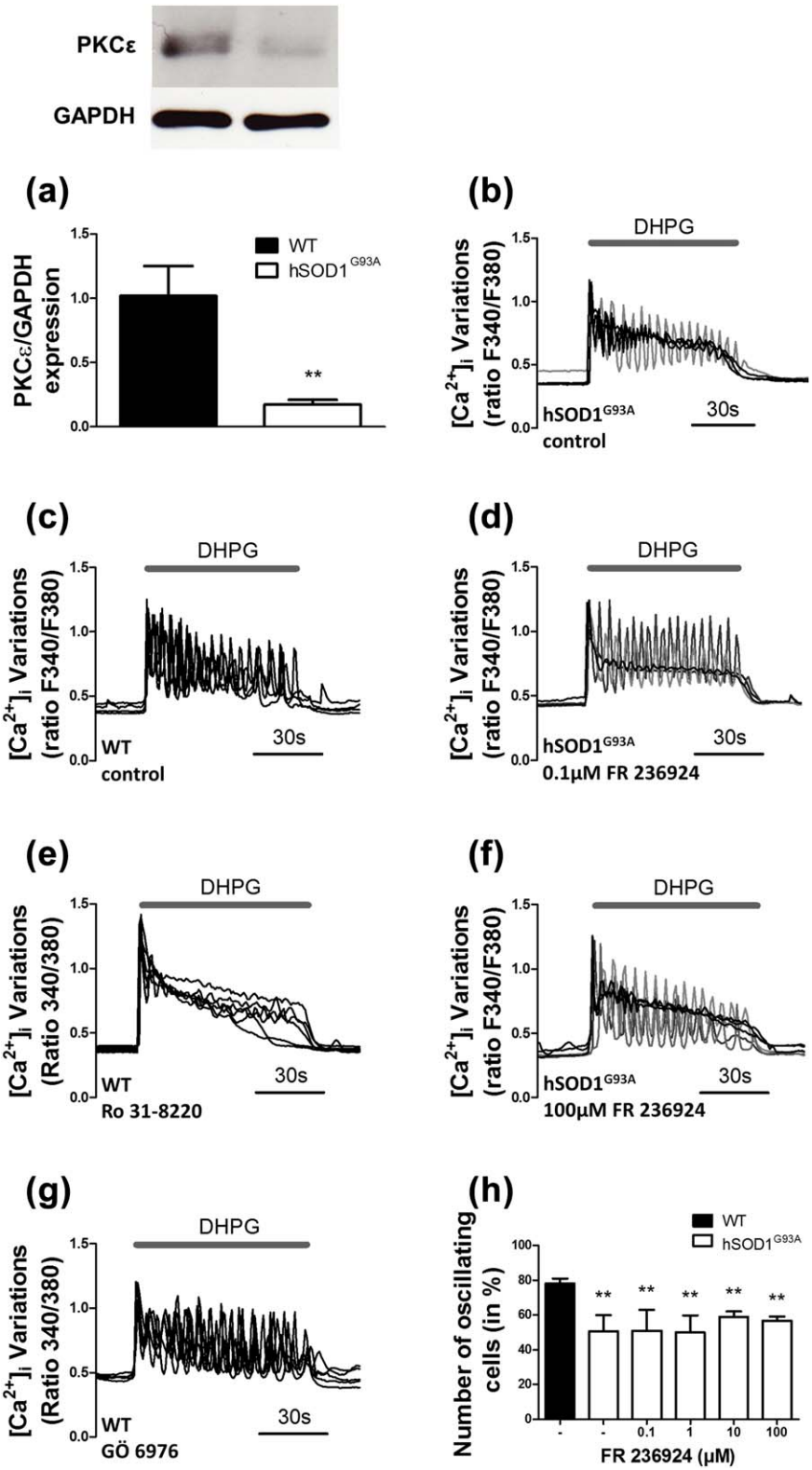

FIGURE 2 Effect of PKC modulation on the mGluR5 calcium signaling in cortical astrocytes. Expression of PKCε (a) was examined by immunoblotting and blots shown are representative of five independent experiments. Histograms show means with *SEM* of five different experiments. After normalization to GAPDH expression, data were shown as relative to the signal obtained in WT astrocytes. Statistical analyses were performed through a *t* test. Astrocytes derived from WT animals were pre-treated for 30 min with medium (b), 5 μM Ro 31–8220 (c) or 10 μM Gö 6979 (d) and Ca^{2+}mobilization was monitored during the exposures to 50 μM DHPG. Calcium mobilization was also monitored in astrocytes derived from $hSOD1^{G93A}$ animals pre-treated for 30 min in control condition (e) or with 0.1 μM (f) to 100 μM (g) FR236924. The proportion of astrocytes showing Ca^{2+} oscillations was quantified and expressed as mean with *SEM* of data from three different experiments (h). Statistical analyses were performed through a one-way ANOVA followed by a Dunnett's test for multiple comparisons (**$P < .01$) [Color figure can be viewed at wileyonlinelibrary.com]

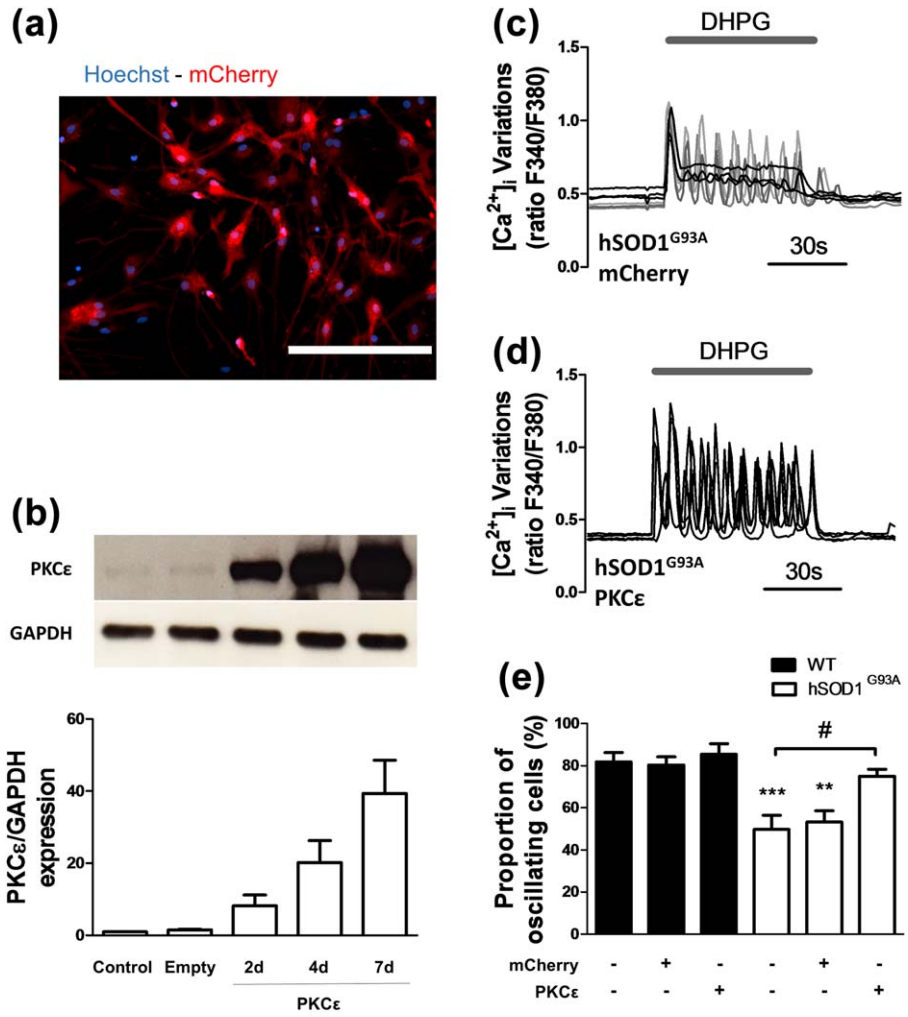

FIGURE 3 Increasing PKCε expression modifies the mGluR5-mediated Ca^{2+} signaling in hSOD1^{G93A} astrocytes. Astrocytes derived from hSOD1^{G93A} rats were exposed for 2, 4, or 7 days with PKCε-lentivirus or with a control lentivirus for 7 days and PKCε expression was measured by immunoblotting (b). Histograms represent mean ± *SEM* of three different experiments. The proportion of mCherry-positive cells was monitored to evaluate the efficiency of infection after 4 days of lentivirus exposure (a). Astrocytes were exposed to 50 μM DHPG for 60 s and cytosolic Ca^{2+} mobilization was monitored. Representative traces indicating calcium responses in different cells are shown from hSOD1^{G93A} astrocyte exposed for 4 days to control lentiviruses (c) or PKCε lentiviruses (d). Panel e illustrates the proportion of cells showing calcium oscillations after infection with control or PKCε lentiviruses. Histograms represent mean ± *SEM* from at least four different experiments. Statistical analyses were performed through a one-way ANOVA followed by a Bonferroni's multiple comparison test (#$P < .05$, **$P < .01$, ***$P < .001$) [Color figure can be viewed at wileyonlinelibrary.com]

Considering the low PKCε expression in hSOD1^{G93A} astrocytes, a lentivirus coding for rat PKCε was designed. The lentivirus also encodes for mCherry expression, facilitating the monitoring of cell infection. Astrocytes derived from hSOD1^{G93A} rats were incubated for 4 days with a control lentivirus (lacking the PKCε sequence) or with the PKCε-lentivirus construct and microscopic evaluation of mCherry fluorescence revealed a high infection efficiency with approximatively 94.15% ± 2.78% mCherry-positive cells, demonstrating a rather homogeneous signal intensity (Figure 3a). PKCε expression in astrocytes infected for 2, 4, or 7 days was examined by immunoblot analysis, revealing 10, 20, and 40 fold greater expression level respectively than was observed in non-infected cells (Figure 3b). As anticipated, infected with the control lentivirus had no effect on PKCε expression levels in both WT and hSOD1^{G93A} astrocytes (data not shown). Astrocytes maintained for 30 days in culture after infection remained positive for mCherry fluorescence, indicating stable viral transduction (data not shown).

To examine whether the lentiviral-mediated increase in PKCε expression could restore the oscillatory-type Ca^{2+} response in hSOD1^{G93A} astrocytes, single cell Ca^{2+} imaging was performed in lentivirus-infected cells. Infection of WT astrocytes with control-lentiviruses or with PKCε-lentiviruses did not change the proportion cells showing Ca^{2+} oscillations upon mGluR5 activation compared with non-infected cells (Figure 3e). Infection of hSOD1^{G93A} astrocytes with control-lentiviruses also did not modify the proportion of cells showing Ca^{2+} oscillations in response to DHPG as compared with non-infected astrocytes (Figure 3c,e). However, increasing the PKCε expression considerably changed the response in hSOD1^{G93A} astrocytes (Figure 3d), with the majority of cells showing Ca^{2+} oscillations upon DHPG exposure (Figure 3e; $P = .015$).

3.3 | Increasing PKCε expression in astrocytes derived from hSOD1^{G93A} astrocytes restores the regulation of glutamate transport following DHPG exposure

In accordance with our previous studies, aspartate uptake measurements revealed that hSOD1^{G93A} astrocytes displayed reduced glutamate transport activity when compared with astrocytes derived from WT animals (Figure 4a,b). In order to compare the regulation of glutamate transporter activity by mGluR5 in astrocytes derived from these two strains, cells were briefly (6 min) exposed to DHPG and resulting aspartate uptake was evaluated immediately. These experiments revealed that exposure of WT astrocytes to 50 μM DHPG induced an increase in the maximal aspartate uptake velocity from 9.84 ± 0.33 to 14.38 ± 0.37 nmol/min/mg of proteins (Figure 4a; $P < .001$). Pretreatment with the PKC inhibitors Gö 6976 or Ro 31–8220 had no effect on aspartate uptake by non-exposed WT astrocytes (Figure 4a). In contrast, DHPG-exposed astrocytes showed a significant increase in the Vmax when pretreated with Gö 6976 ($P < .001$), but no differences were observed when treated with Ro 31–8220 (Figure 4a). Treatment of hSOD1^{G93A} cultures with DHPG induced a modest but significant up-regulation of aspartate uptake velocity from 8.27 ± 0.28 to 9.87 ± 0.30 nmol/min/mg of proteins; $P = .0096$; Figure 4b). Pretreatment of hSOD1^{G93A} astrocytes with Ro 31–8220 totally inhibited the DHPG-mediated up-regulation of uptake while Gö 6976 had no effect (Figure 4b).

The possibility to promote the mGluR5-mediated regulation of glutamate transport in astrocytes by reinforcing the activity of PKCε was investigated through pharmacological and genetic approaches. Wild-type astrocytes treated for 30 min with the specific PKCε activator, FR 236924, exhibited the same glutamate uptake velocity compared with non-treated cells (Figure 4c). This treatment was also without effect on the DHPG-mediated aspartate uptake on both WT and hSOD1^{G93A} astrocytes compared with those only exposed to DHPG (Figure 4c).

In the following experiment, astrocytes were infected for 4 days with control lentiviruses or PKCε-lentiviruses. Astrocytes derived from WT animals or hSOD1^{G93A} animals infected with either viruses exhibited aspartate uptake velocities that did not differ significantly from the corresponding non-infected cells (Figure 4e). The infection with either lentiviruses was also without effect on the DHPG-mediated upregulation of aspartate uptake in WT astrocytes. In contrast, hSOD1^{G93A} astrocytes exposed to DHPG and infected with the PKCε-lentiviruses exhibited a significant increase in the aspartate uptake velocity (Vmax of 14.14 ± 0.53 nmol/min/mg of proteins; $P < .001$), reaching similar values to those observed in WT astrocytes exposed to DHPG (Vmax of 14.52 ± 1.20 nmol/min/mg of proteins; Figure 4e).

3.4 | Knocking-down PKCε expression in astrocytes from WT rats impairs mGluR5-mediated regulation of glutamate transporter activity

Astrocytes derived from WT animals were infected for 4 days with either scrambled-shRNA or PKCε-shRNA lentiviruses. Microscopic detection of E2-Crimson fluorescence revealed a high infection efficiency with approximatively 98.5% ± 2.0% positive cells (data not shown). Infection of WT astrocytes with scrambled-shRNA lentiviruses had no effect on PKCε expression. In contrast, infection with PKCε-shRNA lentiviruses reduced the PKCε expression by 61.61% ± 8.69% (Figure 5a; $P < .001$). Such knocked-down cells had similar PKCε expression levels as those observed in hSOD1^{G93A} astrocytes. The infected cells were also examined for mGluR5 expression by immunoblotting, revealing that altering the expression of PKCε had no impact on mGluR5 expression (Figure 5b).

We further investigated the impact of manipulating PKCε expression on mGluR5-associated Ca^{2+} signaling. Infection of astrocytes with PKCε-shRNA lentiviruses resulted in a decrease in the proportion of cells showing Ca^{2+} oscillations upon exposure to DHPG, and a corresponding increase in cells showing the peak-plateau type Ca^{2+}response (Figure 5d,e; $P < .001$). Infection with scrambled-shRNA lentiviruses had no effect on mGluR5 Ca^{2+} signaling (Figure 5e), resulting in the majority of astrocytes displaying the typical oscillatory type Ca^{2+} response (Figure 5c).

In order to study the influence of PKCε expression on mGluR5-mediated regulation of glutamate transporters, astrocytes were infected with shRNA constructs before monitoring aspartate uptake activity. In the absence of DHPG, uptake measurements revealed identical aspartate uptake in astrocytes infected with scrambled or with PKCε-shRNA lentiviruses (Figure 5f). In contrast, knocking-down PKCε expression considerably impaired the regulation of aspartate uptake induced by DHPG exposure. Thus, while exposure of astrocytes to DHPG considerably promoted aspartate uptake, cells infected with PKCε- shRNA did not show such response to DHPG (Figure 5f; $P < .001$). In control experiments using cells infected with scrambled-shRNA lentivirus, the up-regulation of aspartate uptake following the application of DHPG was totally preserved (no significant difference with non-infected cells, Figure 5f).

4 | DISCUSSION

Recognized as a non-cell-autonomous neurodegenerative disease, ALS is characterized by the progressive loss of motor neurons, a process that involves alterations in both neurons and in surrounding non-neuronal cells (particularly astrocytes). Deletion of mutant SOD1 specifically in astrocytes has been shown to delay microglial activation, disease onset and progression (Yamanaka et al., 2008; Wang, Gutmann, & Roos, 2011). Also, transplantation of glial-restricted precursors that are capable of differentiating into astrocytes harboring hSOD1^{G93A}, induced host motor neuron death, motor and respiratory dysfunctions and reduced GLT-1 expression in WT animals (Papadeas, Kraig, O'banion, Lepore, & Maragakis, 2011). Besides neuronal degeneration, astrocytes located in the neighborhood of motor neurons also exhibited degenerative features in the hSOD1^{G93A} mouse model (Rossi et al., 2008). Since astrocytes are known to participate in the metabolic support of neurons, in the control of neurotransmitter and ion levels in synaptic transmission (Sofroniew & Vinters, 2010), impairment of their function inevitably exacerbates neuronal death in complex diseases such as ALS.

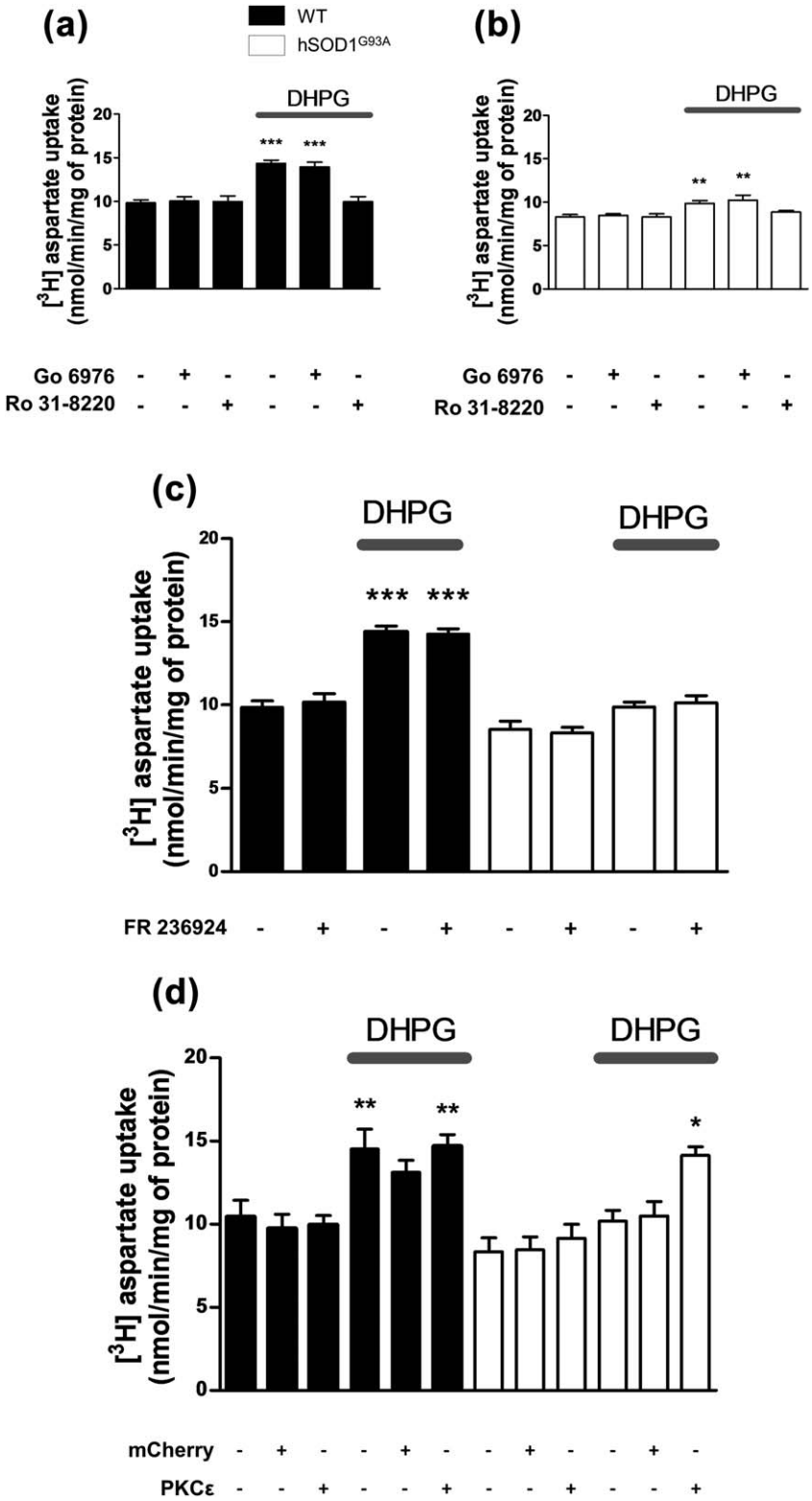

FIGURE 4 Effect of PKCε expression on mGluR5-mediated regulation of glutamate transport in astrocytes derived from hSOD1^{G93A} rats. Both astrocytes derived from WT and hSOD1^{G93A} rats were exposed for 6 min with [^{3}H] D-aspartate and uptake was quantified. Effect of DHPG (50 μM, 15 s before and during the uptake assay) and PKCs inhibitors on the aspartate uptake was evaluated in WT (a) and hSOD1^{G93A} (b) astrocytes. Influence of PKCε activator on DHPG-mediated regulation of aspartate uptake was evaluated on both types of astrocytes (c). Astrocytes were exposed for 4 days with control and PKCε lentiviruses and the effect on the DHPG-mediated regulation of aspartate uptake was evaluated (d). Histrograms represent the mean of the maximal velocity uptake ± the *SEM* of at least three different experiments. Statistical analyses were performed through a one-way ANOVA followed by a Dunnett's multiple comparison test (*$P < .05$, **$P < .01$, ***$P < .001$)

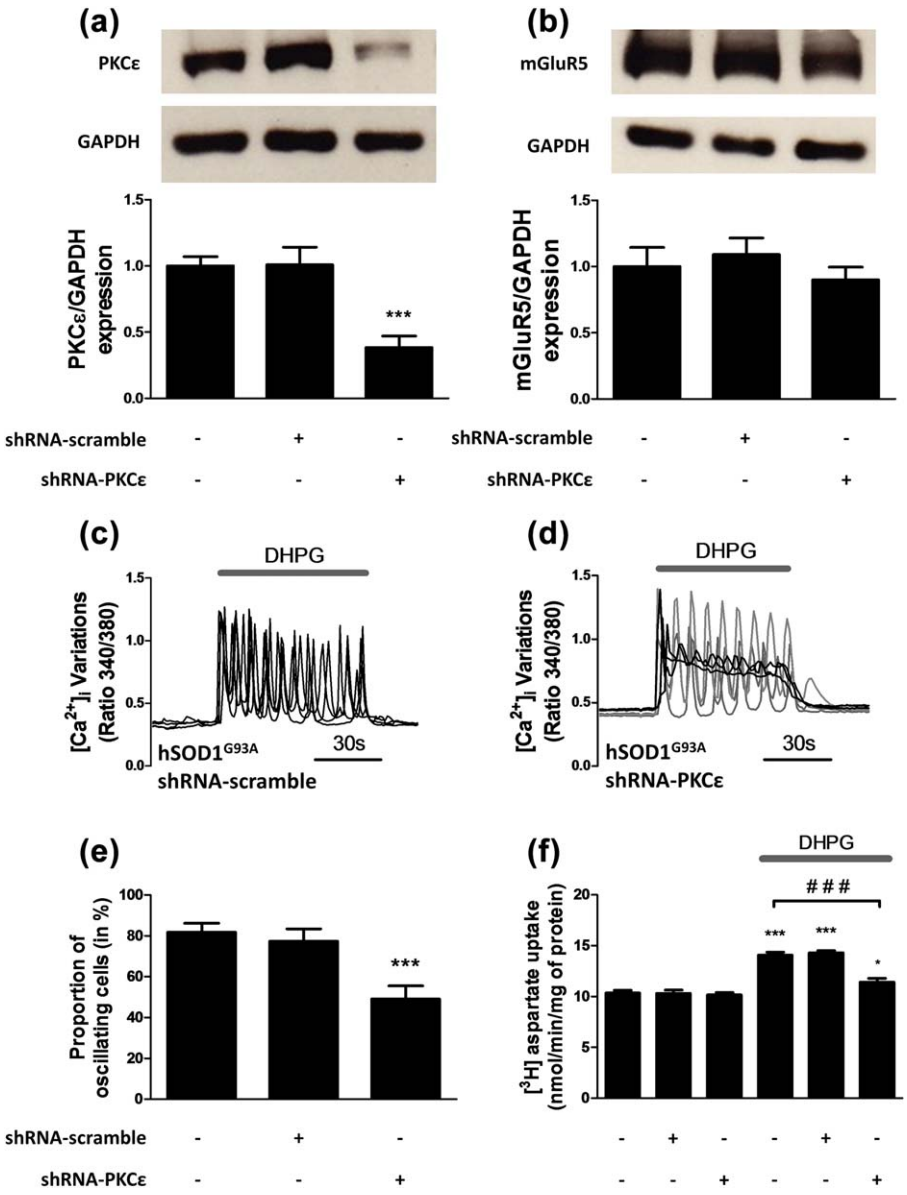

FIGURE 5 Effect of PKCε knockdown on mGluR5-mediated Ca^{2+} signaling and regulation of glutamate transport in astrocytes derived from WT rats. Astrocytes were infected for 4 days with a scrambled shRNA or a shRNA directed against PKCε and PKCε expression (a) or mGluR5 expression (b) was evaluated by immunoblotting. Blots shown are representative of three independent experiments that were all included for densitometric analyses of immunoreactive bands. Histograms show means with *SEM* normalized to GAPDH and expressed as relative to the signal obtained for non-infected cells. Astrocytes were exposed to 50 μM DHPG for 60 s and cytosolic Ca^{2+} mobilization was monitored using Fura2. Representative traces indicating calcium responses from four to six different cells are shown from astrocytes cultures infected with scrambled lentiviruses (c) or with lentiviruses coding for PKCε-shRNA (d). Panel e illustrates the proportion cells showing calcium oscillations. Histograms show means with *SEM* of at least four different experiments. The DHPG-mediated regulation of aspartate uptake was also evaluated after astrocytes infection (f). Histrograms represent the mean of the maximal uptake velocity ± *SEM* from at least three different experiments. Statistical analyses were performed through a one-way ANOVA followed by a Dunnett's (a, b, and c) or a Bonferroni's (f) multiple comparison test (*$P < .05$, ***$P < .001$, ###$P < .001$) [Color figure can be viewed at wileyonlinelibrary.com]

Previous studies have identified mGluR5 in molecular mechanisms leading to astroglial damage. For example, activation of mGluR5 in $hSOD1^{G93A}$-expressing astrocytes induced toxic effects (Rossi et al., 2008). In addition, dying astrocytes have been shown to be strongly immunoreactive for mGluR5 in the spinal cord of symptomatic $hSOD1^{G93A}$ mice and autoptic sALS patients (Aronica, Catania, Geurts, Yankaya, & Troost, 2001). Similar observations have been reported in other models of neurological diseases or in cultured astrocytes exposed to metabolic stress (Paquet et al., 2013). An increased expression of mGluR5 has also been identified in young pre-symptomatic ALS animals, indicating that the receptor likely plays a major role during the early stages of the disease (Martorana et al., 2012). It is, however, noteworthy that in the cell cultures, derived from newborn WT or $hSOD1^{G93A}$ rats, no differences in mGluR5 expression could be detected.

It has been suggested that mGluR5 receptors contribute to the regulation of synaptic activity and to the modulation of neuronal growth, neuroprotection and excitotoxicity (Viwatpinyo & Chongthammakun, 2009). Indeed, it has already been shown that mGluR5 activation leads to a number of effects such as astrocytic proliferation (Kanumilli & Roberts, 2006), the release of brain-derived neurotrophic factor (BDNF; Jean et al., 2008) as well as glio-transmitters such as ATP and glutamate (Bezzi & Volterra, 2014; Panatier et al., 2011), increased glutamate uptake capacity (Aronica et al., 2003; Vermeiren et al., 2005) and modulation of inflammatory responses (Shah, Silverstein, Singh, & Kumar, 2012). Considering the privileged coupling with Gq/11 proteins, intracellular Ca^{2+} mobilization is likely to be involved in the molecular signaling pathways linking the activation of mGluR5 and the regulation of the above glial activities. In healthy astrocytes, the activation of mGluR5, triggers the formation of inositol triphosphate and the subsequent release of intracellular Ca^{2+} in a typical oscillatory pattern (Zur & Deitmer, 2006; Bradley & Challiss, 2011). However, in pathological conditions such as ALS, mGluR5-mediated Ca^{2+} signaling has been shown to be altered (Martorana et al., 2012; Vermeiren et al., 2006), an observation that was supported in the present study by the large proportion of hSOD1^{G93A} astrocytes that switched to a peak/sustained plateau type response upon mGluR5 activation. Interestingly, rescuing the Ca^{2+} oscillations through an interaction with the BH4-domain of Bcl-X_L in hSOD1^{G93A} mice, reduced astrocytic degeneration, slightly postponed disease onset and improved both motor performance and survival of transgenic mice (Martorana et al., 2012). Furthermore, administration of the mGluR5 antagonist MPEP was shown to slow astrocyte degeneration, delaying disease onset and extending the survival of hSOD1^{G93A} mice (Rossi et al., 2008).

The mobilization of Ca^{2+} by mGluR5 is largely dependent on the initial release of Ca^{2+} from intracellular stores, suggesting that downstream signaling effectors of mGluR5 could be implicated in the altered mGluR5 signaling in hSOD1^{G93A} astrocytes. Morita et al. have shown that blockade of PKCs by a series of pharmacological inhibitors converts glutamate-induced Ca^{2+} oscillations to sustained or non-oscillatory responses in astrocytes (Morita, Nakane, Maekawa, & Kudo, 2015). More specifically, reduced expression of the PKCε isoform has been shown to convert the mGluR5 oscillatory profile into a peak-plateau type response (Bradley & Challiss, 2011; Vergouts et al., 2017). Modification in the expression of several protein kinases and phosphatases in the CNS of hSOD1^{G93A} animals has already been reported (Hu, Chernoff, Pelech, & Krieger, 2003). An altered expression of atypical PKCs has been observed in the spinal cord of mice harboring the G93A mutation (Tury, Tolentino, & Zou, 2014). In the present study, a considerable down-regulation of PKCε was observed in hSOD1^{G93A} astrocytes when compared with WT astrocytes. Rescuing PKCε expression in hSOD1^{G93A} astrocytes efficiently promoted the return of typical Ca^{2+} oscillations upon mGluR5 activation. We also demonstrated that silencing PKCε expression in WT astrocytes reduced the number of cells showing mGluR5-mediated Ca^{2+} oscillations. The present data, therefore, suggests that an intrinsic deficiency of PKCε in hSOD1^{G93A} astrocytes may account for the altered signaling profile associated with mGluR5 activation.

For several years, glutamate-mediated excitotoxicity has been considered as one of the molecular mechanisms participating in the onset and progression of ALS. Indeed, deficient glutamate clearance has been reported in synaptosomes prepared from several affected brain structures and spinal cords derived from sporadic ALS patients (Rothstein, Martin, & Kuncl, 1992; Grosskreutz, Van Den Bosch, & Keller, 2010). It has been suggested that this was due to the selective loss of the EAAT2 astroglial glutamate transporter (i.e. the human equivalent of GLT-1), as has been observed in the motor cortex and spinal cord of familial and sporadic ALS patients (Rothstein, Van, Levey, Martin, & Kuncl, 1995; Fray et al., 1998; Grosskreutz et al., 2010). Earlier publications from our group (Dumont, Goursaud, Desmet, & Hermans, 2014; Goursaud, Maloteaux, & Hermans, 2009) and the present results also support such suggestions by showing a reduced aspartate uptake velocity in hSOD1^{G93A} astrocytes. In previous studies, we have proposed that, in hSOD1^{G93A} astrocytes, a shift from an oscillatory profile of mGluR5 signaling to a peak plateau profile was correlated with a loss of mGluR5-mediated regulation of glutamate transporters (Vermeiren et al., 2006). The loss of mGluR5-mediated Ca^{2+} signaling through PKCε dysregulation could, therefore, contribute to the glutamate-induced excitotoxicity observed in ALS. To consolidate this hypothesis, we assessed the role of PKCε on mGluR5-mediated glutamate uptake in both WT and hSOD1^{G93A} astrocytes. Thus, inhibition of all PKCs abolished mGluR5-mediated regulation of aspartate uptake in both WT and hSOD1^{G93A} astrocytes while selective inhibition of novel PKCs had no effect on aspartate uptake following mGluR5 activation. Using lentiviral vectors, it was possible to demonstrate that PKCε transduction could efficiently rescue the deficient mGluR5-mediated regulation of aspartate uptake in hSOD1^{G93A} astrocytes. Knocking down PKCε expression in WT astrocytes also reduced mGluR5 mediated upregulation of aspartate uptake. Interestingly, it has recently been reported that PKCε is markedly reduced in the brain of Alzheimer's disease patients (Khan et al., 2015). It is noteworthy that several studies have reported on a reduced astrocytic glutamate uptake in both patient brain samples and in animal models of Alzheimer's disease (Matos, Augusto, Oliveira, & Agostinho, 2008; Li, Mallory, Alford, Tanaka, & Masliah, 1997; Scott, Gebhardt, Mitrovic, Vandenberg, & Dodd, 2011). The present data were obtained from experiments conducted on astrocytes derived from newborn rodents. When maintained under the present tissue culture conditions, these cells typically adopt features of reactive astrocytes, as found in disease conditions. In this context, it is worth mentioning that hSOD1^{G93A} astrocytes reproduce some of the biochemical alterations observed in ALS. Nevertheless, future *in vivo* studies should help to consolidate the key role played by PKCε in astrocytes.

Apart from a role in mGluR5-mediated Ca^{2+} signaling that regulates glutamate uptake, PKCε also participates in the control of a large variety of intracellular signaling partners (Newton & Messing, 2010). In particular, astrocytic PKCε has been identified as a key regulator of voltage-dependent calcium channels (Burgos et al., 2007). While the present study has focused on mGluR5, PKCε could also control other GPCR signaling pathways, for example those involving purinergic, muscarinic or adrenergic receptors. PKCε target proteins also include key

molecules that orchestrate proinflammatory processes commonly observed in neurological disorders (Burgos et al., 2011). The loss of PKCε documented in the present study could, therefore, also account for other mechanisms involved in the progression of ALS. In this context, a critical role for PKCε has been demonstrated in the control neurites outgrowth (Chen & Tian, 2011; Shirai, Adachi, & Saito, 2008) and it has been reported that promoting axon regeneration could reduce the rate of skeletal muscle denervation and extend the life span of mice in a model of ALS (Jokic et al., 2006). Another example of the possible influence of PKCε is provided by studies on cell differentiation in which PKCε has been shown to control astrocytic differentiation of neural stem cells (Steinhart, Kazimirsky, Okhrimenko, Ben-Hur, & Brodie, 2007). Indeed, transplantation of neural precursors into the spinal cord of a mouse model of ALS gave rise to GFAP-positive donor cells that provided temporarily extended neuroprotection, improved motor function and prolonged animal survival times (Martin & Liu, 2007). The pathogenesis of ALS is also characterized by mitochondrial dysfunction and energy deficits (VandeVelde et al., 2011; Ioannides, Ngo, Henderson, Mccombe, & Steyn, 2016; Vergouts et al., 2015). PKCδ and PKCε participate in the control of cell energy homeostasis and an imbalance between these two PKCs is thought to disturb the flux of fuel entering the Krebs cycle and the overall production of energy (Gong et al., 2012). Finally, PKCε has also been shown to influence mitochondrial processes that regulate free radical production (Matsuzaki, Szweda, Szweda, & Humphries, 2009) which is of significance since oxidative stress is known to be one of the mechanisms by which motor neuron death occurs in ALS (Barber & Shaw, 2010). In the vast majority of ALS cases, the mechanisms involved in motor neuron degeneration are multi-factorial. Taking together, published data strongly suggests that PKCε alterations could be implicated in the numerous cellular dysfunctionalities that have been observed in ALS. This member of the novel kinase family would therefore represent an interesting target for future therapeutic strategies in neurological disorder.

Data from our previous studies (Vergouts et al., 2017) and the present observations suggest that PKCε indirectly acts as a key regulator in the maintenance of mGluR5-mediated regulation of glutamate clearance by astroglial transporters. A major observation with implications for future research on ALS is that rescuing PKCε expression in hSOD1^{G93A} astrocytes permits the restoration of a dynamic mGluR5-dependent control of glutamate clearance by these cells. As also shown in several disorders such as Huntington's disease, Alzheimer's disease, Parkinson's disease and epilepsy (Dong, Wang, & Qin, 2009; Sheldon & Robinson, 2007), high levels of extracellular glutamate in the tripartite synapse leading to excitotoxicity is a well-documented feature of ALS. Future studies should therefore consider the possibility to directly or indirectly manipulating the expression of PKCε in astrocytes in nervous structures specifically affected at the onset of the disease in order to potentially limit the damages caused by excitotoxic insults.

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