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Low doses of Perampanel protect striatal and hippocampal neurons against in vitro ischemia by reversing the ischemia-induced alteration of AMPA receptor subunit composition

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ABSTRACT

Energy depletion caused by ischemic brain insults may result in persistent neuronal depolarization accompanied by hyper-stimulation of ionotropic glutamate receptors and excitotoxic phenomena, possibly leading to cell death. The use of glutamate receptor antagonists, such as the AMPARs antagonist Perampanel (PER), might be a pharmacological approach to counteract the excessive over-activation of glutamate receptors providing neuroprotective effects. Using electrophysiological and molecular analyses, we investigated the effect of PER against in vitro ischemia obtained by oxygen and glucose deprivation (OGD) in rat slices of two brain structures particularly sensitive to ischemic insults, the nucleus striatum and the hippocampus. We found that in these regions PER was able to avoid the OGD-induced neuronal suffering, at low doses not reducing basal excitatory synaptic transmission and not altering long-term potentiation (LTP) induction. Furthermore, in both the analysed regions, PER blocked a pathological form of LTP, namely ischemic LTP (iLTP). Finally, we hypothesized that the protective effect of PER against OGD was due to its capability to normalize the altered synaptic localization and function of AMPAR subunits, occuring after an ischemic insult. Taken together these findings support the idea that PER is a drug potentially effective to counteract ischemic damage.

1. Introduction

Energy depletion, caused by reduced blood flow to the central nervous system (CNS), is responsible for diminished supplement of oxygen, glucose and other substances to neurons, leading to persistent neuronal depolarization accompanied by increased glutamate release in the extracellular space (Dirnagl et al., 1999).

The subsequent activation of synaptic and non-synaptic glutamate receptors has been suggested to initiate excitotoxic cascades representing a major key pathogenic event in ischemic neuronal death (Bano and Nicotera, 2007; Kwak and Weiss, 2006; Lai et al., 2014; Lipton, 1999).

N-methyl-d aspartic receptors (NMDARs) and α-amino-3-hydroxy-5methyl-4-isoxazole propionate receptors (AMPARs) seem to be strongly implicated in the ischemic-related excitotoxic pathway. In fact, many studies reported that after an ischemic insult these receptors display altered expression and function (Arundine and Tymianski, 2004; Lea and Faden Lea and Faden, 2001). The ischemic insult leads to increased GluA1/GluA2 AMPAR subunit ratio, which allows massive Ca^{2+} entry through the receptor (Pellegrini-Giampietro et al., 1997). The AMPAR has also a crucial role in the induction and maintenance of long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity that are thought to represent the basis of memory and learning processes (Malinow and Malenka, 2002). LTP requires changes in the number and function of glutamate receptors, including synthesis and insertion in the postsynaptic membrane of new AMPARs to ease the potentiation of synaptic transmission (Buonarati et al., 2019). The relation occurring among ischemic insult, change of

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glutamate neurotransmission and synaptic plasticity, is suggested by the evidence that a brief oxygen and glucose deprivation (OGD) is able to induce a pathological form of synaptic plasticity named ischemic long-term potentiation (iLTP) (Calabresi et al., 2002). This aberrant form of synaptic plasticity has been considered the electrophysiological correlate of the molecular alterations taking place during apoptotic cell death, with still unknown functional consequences (Calabresi et al., 2003). Thus, during pathological conditions characterized by limited neuronal energetic supply, the consequent altered neuronal synaptic plastic properties could negatively impair the cognitive abilities (Delcasso et al., 2014) and brain coping strategies of patients. A hypothetical optimal neuroprotective strategy against brain ischemia, preserving neuronal structure and/or function (Wiendl et al., 2015), should counteract the activation of the glutamate-triggered excitotoxic pathways without impairing the capability of neurons to express physiological synaptic plasticity. Several glutamate receptors antagonists have been tested as neuroprotective compounds against brain ischemia (Hanada et al., 2011). AMPARs antagonists, such as Talampanel and other compounds, have also been considered for their potential ability to attenuate brain damage after focal or cerebral ischemia (Meldrum and Rogawski, 2007). However, the use of AMPAR antagonists in a clinical setting could be limited by many short-comings, such as poor solubility, short half-life and precipitation in the kidney (Hanada et al., 2011).

Perampanel (PER) is an AMPAR antagonist, unique for its capability to cross the blood brain barrier with respect to other AMPAR antagonists, together with a good oral bioavailability and favourable pharmacokinetic properties (Rogawski and Hanada, 2013). Thus, due to these properties and the mechanism of action antagonizing glutamate transmission, it is possible to hypothesize a beneficial neuroprotective effect of PER against energetic failure, that will preserve neuronal transmission. Following this hypothesis, we analysed the potential protective effect of PER in the nucleus striatum and in the hippocampus, two brain structures particularly vulnerable to ischemic insults and of pivotal importance for different forms of learning and memory. Furthermore, we tested whether the doses of PER able to counteract the detrimental effects of OGD were able to influence the expression of synaptic plasticity, as well as the postsynaptic localization of AMPA receptor subunits in both of the analysed regions.

2. Materials and methods

All experiments were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/ECC), in accordance with a protocol approved by the Animal Care and Use Committee at the University of Perugia and with the ARRIVE guidelines. All efforts were made to minimize the number of animals used and their suffering.

2.1. Electrophysiology

Corticostriatal and hippocampal slices (thickness 270 and 400 μm respectively) were cut from brains of four weeks old Wistar male rats (Charles River, Italy) using a vibratome. All slices were submerged in an artificial cerebrospinal fluid (ACFS) or Kreb's solution, bubbled with 95% O_2, 5% CO_2 gas mixture at 32 °C for cortical striatal slices and 29 °C for hippocampal slices (flow rate 2.9–3.3 ml/min). The composition of the Kreb's solution was (in mmol/l) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 10 Glucose and 25 $NaHCO_3$. Each single striatal or hippocampal slice was transferred to a recording chamber and submerged in a continuously flowing Kreb's solution for both extracellular and patch-clamp recordings. For extracellular recordings an Axoclamp 2B amplifier (Molecular Devices, USA) was used.

Hippocampal extracellular *excitatory post-synaptic potentials* (fEPSPs) were evoked every 10 s with a stimulating bipolar electrode placed in the Schaffer collateral fibers (10 μs duration; 30–50 V intensity) and recorded with a glass microelectrode filled with 2 mol/l NaCl placed in the *stratum radiatum* of the CA1 hippocampal region. Striatal fEPSPs were evoked every 10 s by stimulating the cortical glutamatergic afferents to the dorsolateral striatum.

In vitro ischemia was delivered by switching the standard Kreb's solution to an artificial cerebrospinal fluid solution gassed with 95% N_2 and 5% CO_2 in which sucrose replaces glucose (oxygen and glucose deprivation, OGD). The duration of OGD protocol was region specific, 10 min for the striatum and 8 min for the CA1 hippocampal region (Mazzocchetti et al., 2018).

To induce LTP in the CA1 hippocampal region, a high-frequency stimulation (HFS) protocol consisting of one train lasting 1 s, was delivered at 100 Hz (Kleschevnikov et al., 2004) after acquiring a stable baseline for 10 min. In hippocampal slices, iLTP was induced by a brief OGD episode (2–2.5 min) (Crepel et al., 1993). In particular, for iLTP experiments, fEPSPs were evoked in presence of a Kreb's solution containing 10 μM bicuculline to block $GABA_A$ receptors, 0.3 mM Mg^{2+} to enhance NMDA receptor-mediated response and 10 μM glycine to saturate the glycine allosteric site of the NMDA receptors (Crepel and Ben-Ari, 1996; Crepel et al., 1993).

Whole-cell voltage-clamp recordings were performed on striatal spiny projection neurons (SPNs) (access resistance 15–30 MΩ; holding potential −80 mV) and pyramidal cells (access resistance 6–15 MΩ; holding potential −60 mV) visualized using differential interference contrast (Nomarski) and infrared microscopy (Olympus). Recordings were made with a Multiclamp 700B amplifier (Molecular Devices), using a borosilicate glass pipette (4-7 MΩ) filled with a standard internal solution containing (in mM): 145 K^+-gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 0.1 EGTA, 10 HEPES, 0.3 Na^+-GTP and 2 Mg^{2+}-ATP, adjusted to pH 7.3 with KOH. The membrane potential was not corrected for liquid junction potential.

In order to evoke postsynaptic excitatory currents (EPSCs) from SPNs and pyramidal cell a bipolar stimulating electrode was placed intrastriatal or on Schaffer collateral fibers, respectively. Current-voltage (I-V) relationship of both CA1 pyramidal cells and SPNs was obtained by applying hyperpolarizing and depolarizing steps of current (50 pA, 2 s). All the experiments were conducted in the presence of 50 μM of the GABA receptor antagonist picrotoxin, in order to isolate the glutamate transmission.

In the striatum, LTP was induced by HFS protocol consisting of three trains lasting 3 s (20 s interval) delivered at 100 Hz. During HFS protocol, the stimulus intensity was increased to supra-threshold levels. External Mg^{2+} ions were omitted to maximize the contribution of NMDA receptors during LTP experiments (Calabresi et al., 1992).

The iLTP was induced in corticostriatal slices by a brief OGD episode (2–2.5 min). Also in this case, external Mg^{2+} ions were omitted to maximize the contribution of NMDA receptors during LTP experiments (Calabresi et al., 1992) and the SPNs were clamped to a holding potential of −50 mV.

2.2. Subcellular fractionation and western blotting

For the purification of triton-insoluble postsynaptic fractions (TIF), striatal and hippocampal tissues were homogenized with a Teflon-glass potter in ice-cold buffer containing (in mM) 320 sucrose, 1 Hepes, 1 $MgCl_2$, 1 $NaHCO_3$, 0.1 phenylmethylsulfonylfluoride at pH 7.4 in the presence of Complete™ Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Basel, Switzerland) and phosSTOP™ Phosphatase Inhibitor (Roche Diagnostics). The sample was spun at 13.000 *g* × 15 min at 4 °C. The resulting pellet was resuspended in Triton-KCl buffer (0.5% Triton X-100 and 150 mM KCl) and, after 15 min incubation on ice, it was spun further at 100.000 *g* × 1 h at 4 °C. The pellet (triton-insoluble postsynaptic fraction, TIF) was resuspended in 20 mM Hepes buffer supplemented with Complete™ Protease Inhibitor Cocktail Tablets and stored at −80 °C.

The levels of AMPAR subunits in the homogenate and TIF were

analysed by western blotting. Homogenate and TIF samples were separated onto a 7% acrylamide/bis acrylamide gel. Proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and probed with the appropriate primary antibodies followed by the corresponding HRP-conjugated secondary antibodies. Labeling detection was performed with ChemiDoc MP Imaging System (Bio-Rad) and images were acquired with ImageLab software (Bio-Rad). The primary antibodies used in this study are anti-GluA1 (dilution 1:1000; Neuromab), anti-GluA2 (dilution 1:500; Neuromab), anti-GluA3 (dilution 1:1000; Synaptic System), anti-GluA1p845 (dilution 1:1000; Merck Millipore) and anti-tubulin (dilution 1:20000; Sigma-Aldrich). The latter was always used as loading control for normalization.

2.3. Drugs

Drugs were bath applied by dissolving them to the desired final concentration in the Krebs' solution and by switching the perfusion from control solution to drug-containing solution. PER was kindly provided by EISAI. The in vitro doses of PER were selected according to literature (Ceolin et al., 2012; Hanada et al., 2011). In order to evaluate the effect of PER on I-V relationship and synaptic transmission, PER was applied in the recording chamber for at least 20 min. In OGD experiments PER was delivered on the recording chamber for at least 10 min before OGD and maintained throughout the experiment. During the HFS protocol, PER was maintained throughout all the duration of the recording.

2.4. Statistical analysis

For electrophysiological experiments data analysis was performed off-line using Clampfit 10 (Molecular Devices). Values given in the text and figures are mean ± SEM, n representing the number experiments. Changes of the fEPSP amplitude and slope are expressed as percentage of the baseline, the latter representing the normalized fEPSP or EPSC mean amplitude acquired during a stable period (10–15 min) before the start of the experimental protocols involving PER, OGD and HFS. Two-way ANOVA or the Student's *t*-test was used. For the molecular analysis data were normalized against tubulin and shown in the graph as mean ± SEM. The one-way ANOVA or the Student's *t*-test was used. The significance level was established at *p < .05, **p < .01, ***p < .001.

3. Results

3.1. Effect of PER on synaptic transmission

We tested the effects of PER on the excitatory synaptic transmission by extracellular field potentials recordings in the CA1 hippocampal region and dorsal striatum stimulating, respectively, the Schaffer's collaterals or the corticostriatal glutamatergic fibers. After acquiring stable fEPSPs responses for 10 min, PER was bath applied for 20 min on the slice at different concentrations (striatum 0.1 nM to 1000 nM, hippocampus 1 nM to 3000 nM, Fig. 1A, B). As expected, PER dose-dependently reduced the fEPSP response in both striatum and hippocampus. Specifically, we found that in striatal slices, while 0.1–0.3 nM PER did not reduce the fEPSP amplitude, doses higher than 1 nM were effective, (PER 3 nM, fEPSP amplitude 87.51 ± 2.27%, control vs 3 nM PER (n = 8) Student's *t*-test, p < .01, Fig. 1A). The calculated IC_{50} for the dose-response relationship was 132 nM.

In the hippocampus PER doses lower than 100 nM were unable to reduce the fEPSP slope. In fact, while 1–30 nM PER did not affect the response, in presence of 100 nM PER the fEPSP slope was 80.49 ± 3.10% of the baseline (control vs PER, (n = 5) Student's *t*-test, p < .05, Fig. 1B). In the CA1 hippocampal region, PER induced a dose-dependent reduction of the fEPSP slope with an IC_{50} of 335 nM (Fig. 1B).

3.2. Effect of PER on OGD-induced loss of field potential

We then analysed the possible neuroprotective effect of PER against the alteration of neurotransmission, potentially reflecting neuronal death, induced by in vitro ischemia (OGD) in the nucleus striatum. After recording a stable fEPSP for 10 min, an oxygen and glucose-free solution was applied for 10 min, a time known to produce the irreversible loss of the fEPSP amplitude in this region (Calabresi et al., 2000). The fEPSP amplitude was recorded for additional 30 min both in control experiments or in presence of different concentrations of PER. We found that, ischemic insult irreversibly suppressed the fEPSP both in control conditions and in presence of 0.1 nM PER (control (n = 7) vs PER (n = 6), two-way ANOVA, p > .05; Fig. 2A). Conversely, 0.3 nM and 1 nM PER were able to induce a significant recovery of the fEPSP amplitude, as measured 30 min following OGD (PER 0.3 nM fEPSP amplitude 78.72 ± 8.17% of baseline, control (n = 7) vs PER (n = 7), two-way ANOVA, p < .001; PER 1 nM fEPSP amplitude 85.44 ± 11.55% of baseline, control (n = 7) vs PER (n = 6), two-way ANOVA, p < .001; Fig. 2B, C). These data suggest that, in the nucleus striatum, the neuroprotective effect of PER against in vitro ischemia is dose-dependent, presenting a maximal neuroprotective effect achieved by 1 nM of the drug and a calculated EC_{50} of 0.16 nM (Fig. 2D). In order to test whether the first dose of PER, able to protect neurons against in vitro ischemia, could affect the SPNs membrane excitability, we performed whole-cell patch-clamp recordings of striatal SPNs to measure the firing activity before and 10 min after exposure of the slices to 0.3 nM (Fig. 2E). The calculated I-V curves showed no differences between the recordings obtained in control conditions and in presence of 0.3 nM PER (striatum, control vs 0.3 nM PER (n = 5), two-way ANOVA, p > .05).

Since the CA1 hippocampal region is one of the most vulnerable areas of the brain to ischemia (Pulsinelli, 1985), 8 min OGD are sufficient to produce an irreversible depression of synaptic activity. We found that, in the hippocampal CA1 region, the ischemic insult irreversibly suppressed the fEPSP slope both in control experiments and in presence of 10 nM PER (control (n = 7) vs PER (n = 5), two-way ANOVA, p > .05, Fig. 3A). Conversely, 30 and 100 nM PER significantly recovered the fEPSP slope, as measured 30 min after OGD application (PER 30 nM, fEPSP slope 53.53 ± 9.63% of baseline, control (n = 7) vs PER (n = 6), two-way ANOVA, p < .001, Fig. 3B; PER 100 nM, fEPSP slope 98.87 ± 5.69% of baseline, control (n = 7) vs PER (n = 5), two-way ANOVA, p < .001, Fig. 3C). Interestingly, 100 nM PER caused only a mild reduction of excitatory synaptic transmission (Fig. 1B). The neuroprotective effect of PER was dose-dependent with a calculated EC_{50} of 30.04 nM (Fig. 3D). The calculated I-V curves, obteined through patch-clamp recordings of pyramidal CA1 neurons, showed no differences between the recordings obtained in control conditions and in presence of 30 nM PER (control vs 30 nM PER (n = 4), two-way ANOVA, p > .05; Fig. 3E).

3.3. Effect of PER on physiological and ischemic long-term potentiation of synaptic transmission

Antiepileptic drugs (AEDs) are known to possibly induce memory deficits in patients, even at therapeutic doses, as confirmed also in experimental models (Detrait et al., 2010). Thus, we tested whether PER neuroprotective doses against OGD impaired LTP induction both in the nucleus striatum and hippocampus. To achieve this goal, we recorded striatal EPSCs for SPNs and hippocampal CA1 fEPSPs, for 10 min and induced LTP by HFS of afferent fibers to the striatum or to the CA1 hippocampal region. The EPSCs amplitude measured 30 min after the HFS protocol in the striatum and the fEPSP slopes measured 50 min after the HFS in the hippocampus were not significantly different in control conditions and in presence of PER in both the structures (striatum, EPSCs amplitude in control conditions 158.48 ± 6.16%, in PER 0.3 nM 144.10 ± 11.31%, control (n = 5) vs PER (n = 5), two-

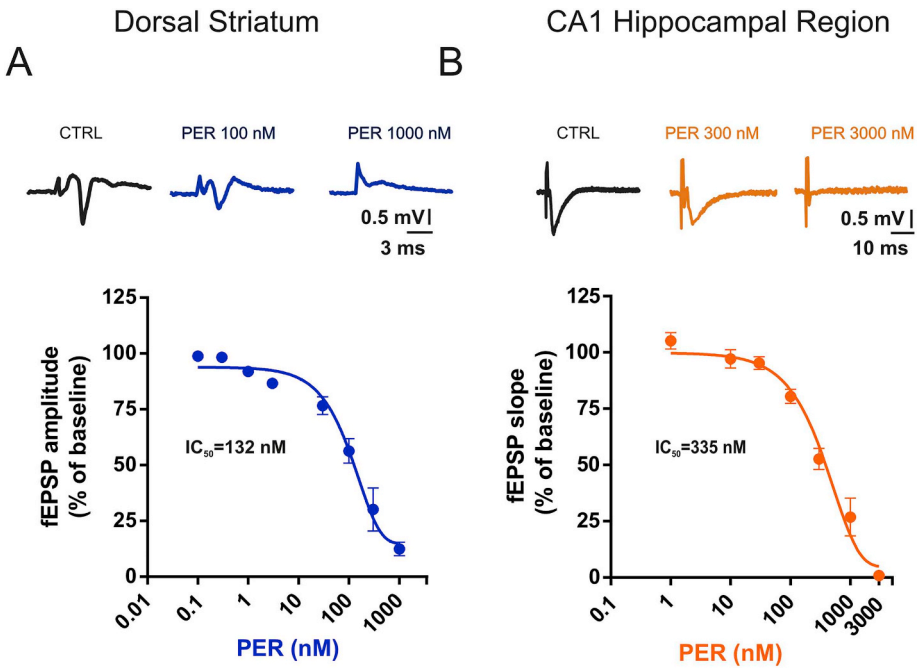

Fig. 1. Effect of PER on physiological synaptic transmission in striatum and CA1 hippocampal region. A) Upper representative traces of striatal fEPSPs recorded in Kreb's solution or in the presence of increasing doses of PER; the graph describes the dose-response curve for PER showing an IC_{50} of 132 nM (control vs 0.1 nM PER (n = 7), p > .05; control vs 0.3 nM PER (n = 11), p > .05, control vs 1 nM PER (n = 12), p > .05; control vs 3 nM PER (n = 8), p < .01; control vs 10 nM PER (n = 6), p < .05, control vs 30 nM PER (n = 9), p < .01, control vs 300 nM PER (n = 6), control vs 1000 nM PER (n = 5), p < .01). B) Upper representative traces of hippocampal fEPSPs. The graph describes the dose-response curve for PER showing an IC_{50} of 335 nM (control vs 1 nM to 30 nM, (n = 5, for each concentration), p > .05; control vs 300 nM PER (n = 5), p < .01, control vs 3000 nM PER (n = 5), p < .001).

way ANOVA, p > .05; Fig. 4A; hippocampus, fEPSP slope in control conditions, 167.76 ± 16.56%, in 30 nM PER, 139.92 ± 9.28%, control (n = 5) vs PER (n = 5), two-way ANOVA, p > .05, Fig. 4B). It should be noted that in the CA1 hippocampal region 100 nM PER, the dose producing maximal protective effect, significantly impaired the LTP (data not shown).

We next tested the effects of PER on iLTP, an aberrant form of synaptic plasticity reflecting metabolic neuronal suffering induced by an ischemic insult. In the nucleus striatum, a brief OGD episode (2–2.5 min) is able to induce a transient depression of the EPSCs of SPNs followed, after washout of the ischemic solution, by reversal of the phenomenon and a subsequent and persistent potentiation of the EPSCs amplitude (EPSCs amplitude 30 min after OGD 155.15 ± 4.28% of baseline; Fig. 4C). We found that the application of 0.3 nM PER prevented the occurrence of iLTP (EPSCs amplitude 85.77 ± 7.78%, control (n = 4) vs PER (n = 4), p < .001, Fig. 4C). As previously shown (Costa et al., 2011), in the hippocampus OGD was also able to induce iLTP (fEPSPs slope 30 min after OGD 140.91 ± 1.90% of baseline) and interestingly, 30 nM PER was able to prevent the induction of the pathological LTP in this region (PER, fEPSPs slope 30 min after OGD 98.37 ± 6.82% of baseline; control (n = 6), vs PER 30 nM (n = 5), p < .001, Fig. 4D).

3.4. Effect of PER on OGD-induced alterations of AMPA receptor subunit composition

We evaluated by western blotting analysis in a total homogenate fraction and in a triton-insoluble post-synaptic fraction (TIF), the possibility that alterations in the levels and subunit composition of AMPAR may be involved in the observed effects induced by OGD and treatment with PER. Analysis of AMPAR subunit levels in total homogenate indicated a higher expression of both GluA1 and GluA2 subunits in the hippocampus compared to the striatum (p < .01, GluA1, hippocampus vs striatum; p < .05, GluA2, hippocampus vs striatum; Fig. 5A).

Moreover, we found that application of the OGD protocol to hippocampal slices induced a significant increase of GluA1 AMPAR subunit synaptic levels (*p < .05, post-OGD vs pre-OGD, Fig. 5B). Notably, concomitant treatment with PER was sufficient to rescue synaptic GluA1 subunit to control levels (#p < .05, post-OGD + PER vs post-OGD, Fig. 5B). Similarly, PER significantly decreased GluA1 synaptic levels in the striatum from OGD-treated corticostriatal slices (#p < .05, post-OGD + PER vs post-OGD, Fig. 5C). No effect on GluA2 and GluA3 subunit levels at synapses was observed after OGD in absence or presence of PER both in hippocampus (Fig. 5B) and in striatum (Fig. 5C). Finally, no alteration of the phosphorylation of GluA1-Ser845, a well-validated marker of synaptic plasticity (Esteban et al., 2003; Hu et al., 2007; Oh et al., 2006), was found both in hippocampus (Fig. 5B) and in striatum (Fig. 5C).

4. Discussion

In this study we explored the possible neuroprotective effect of the AMPAR antagonist PER on energy deprivation induced by bath application of an oxygen- and glucose-free solution (OGD) on rat hippocampal and striatal slices. We found that PER, at doses unable to affect basal neurotransmission and the physiological LTP induction, was able to reduce the detrimental effects of OGD on hippocampal and striatal neurons, including the expression of iLTP, a pathological form of synaptic plasticity. Finally, we hypothesized that, the protective effect of PER could rely on its ability to reduce the increase of GluA1-bearing AMPARs induced by OGD.

The AMPARs antagonist PER at concentrations higher than 30 nM in the striatum and higher 100 nM in the hippocampus, reduce synaptic transmission in a concentration-dependent manner, as previously described (Ceolin et al., 2012). We found that the IC_{50} of PER in the striatum (132 nM) was significantly lower than that measured in the hippocampus (335 nM), highlighting a higher sensitivity of the striatum to the drug with respect to the hippocampus. This could be explained by the different expression of AMPARs in these brain regions, as observed by molecular analysis of AMPARs subunit levels in total homogenate of these tissues.

Moreover, we found that PER exerted a dose-dependent neuroprotective effect against in vitro ischemia obtained by OGD, and it was able to block the pathological iLTP, in both the striatum and hippocampus. Indeed, AMPARs are known to be particularly important for striatal (Di Filippo et al., 2008; Saulle et al., 2002) and hippocampal iLTP (Dias et al., 2013).

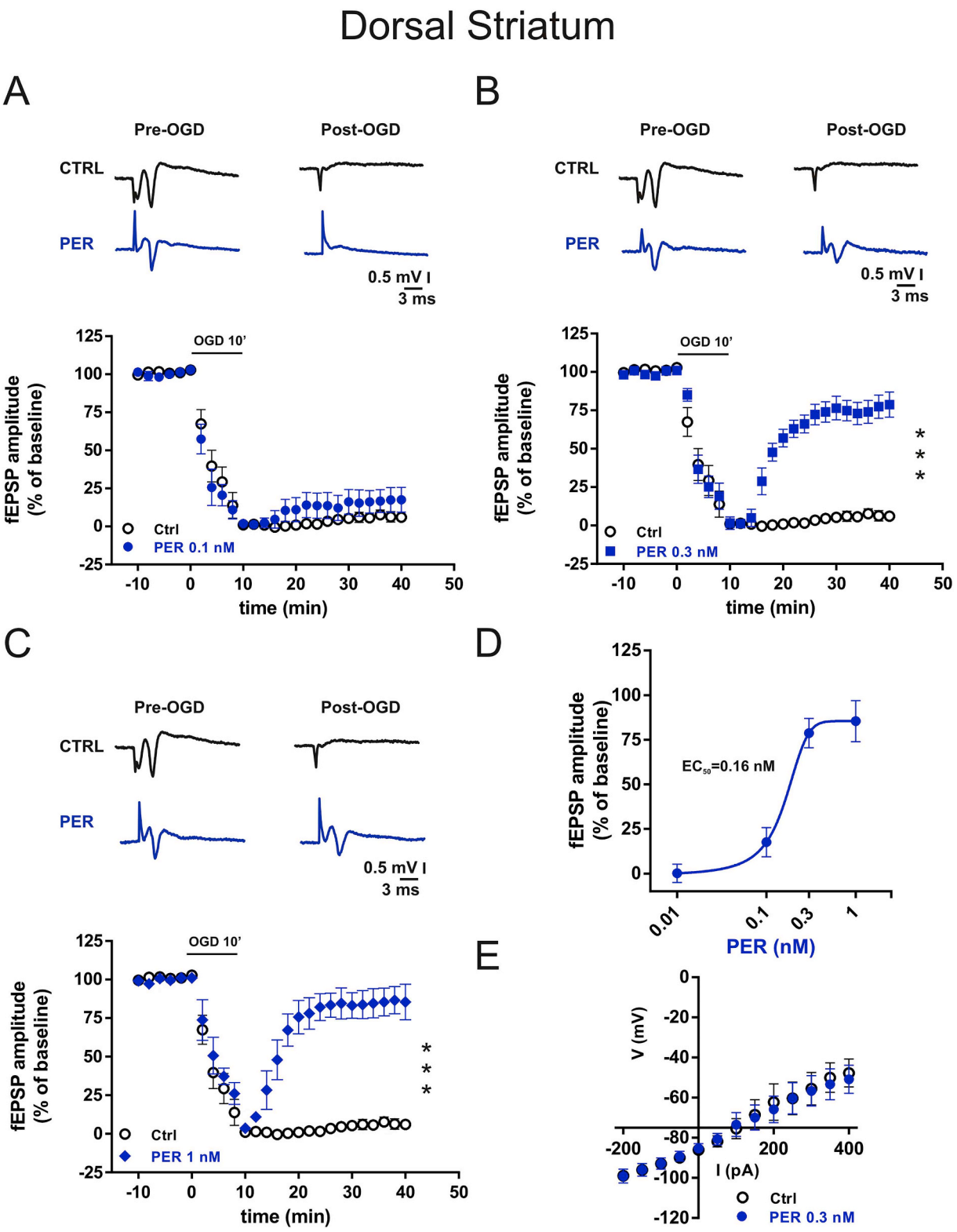

Fig. 2. Neuroprotective effect of PER against OGD in striatum. A-C) Upper representative traces of striatal fEPSPs recorded pre and post-OGD both in control conditions and in presence of increasing doses of PER. The time-course plot shows the effect of OGD in control slices and in slices treated with 0.1, 0.3 and 1 nM PER (control open circle; 0.1 nM PER blue circle; 0.3 nM PER blue square; 1 nM PER blue diamond). D) Dose-response curve of the neuroprotective effect of PER on in vitro ischemia induced by OGD in the striatal region. The EC_{50} is 0.16 nM. E) Input-Output (I/O) curves recorded from striatal neurons show no difference between neurons recorded in control conditions and those treated with PER (striatum, control open circle vs 0.3 nM PER blue circle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

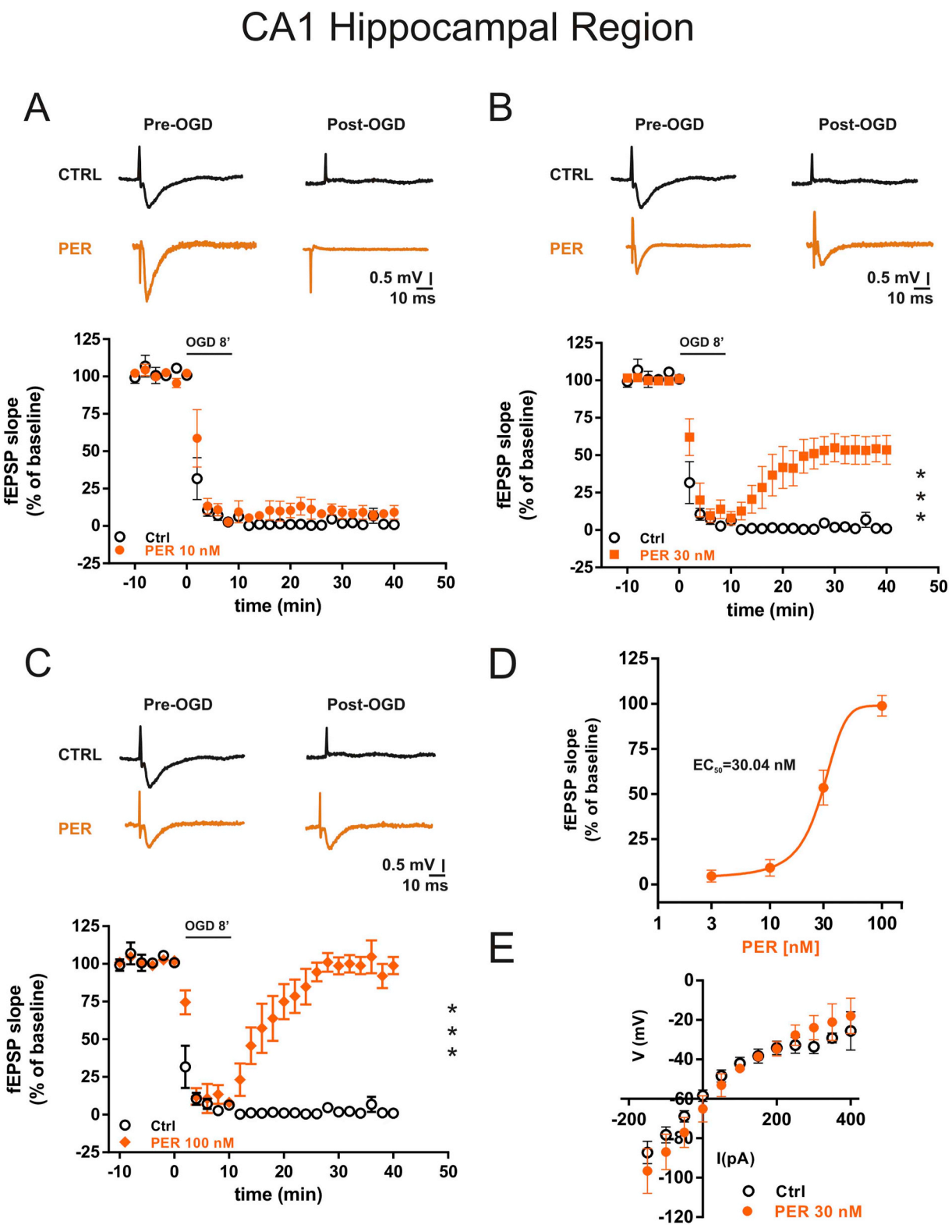

Fig. 3. Neuroprotective effect of PER against OGD in CA1 hippocampal region. A-C) Upper representative traces of hippocampal fEPSPs recorded pre- and post-OGD both in control conditions and in presence of increasing doses of PER. The time-course plot shows the effect of OGD in control slices and in slices treated with 10, 30 and 100 nM PER (control open circle; 10 nM PER orange circle; 30 nM PER orange square; 100 nM PER orange diamond). D) Dose-response curve of the neuroprotective effect of PER on in vitro ischemia induced by OGD protocol. The graph shows that the maximal neuroprotective effect was achieved with 100 nM PER. The EC_{50} was 34.21 nM. E) Input-Output (I/O) curves recorded from pyramidal neurons show no difference between neurons recorded in control conditions and those treated with PER (control open circle vs 30 nM PER orange circle).

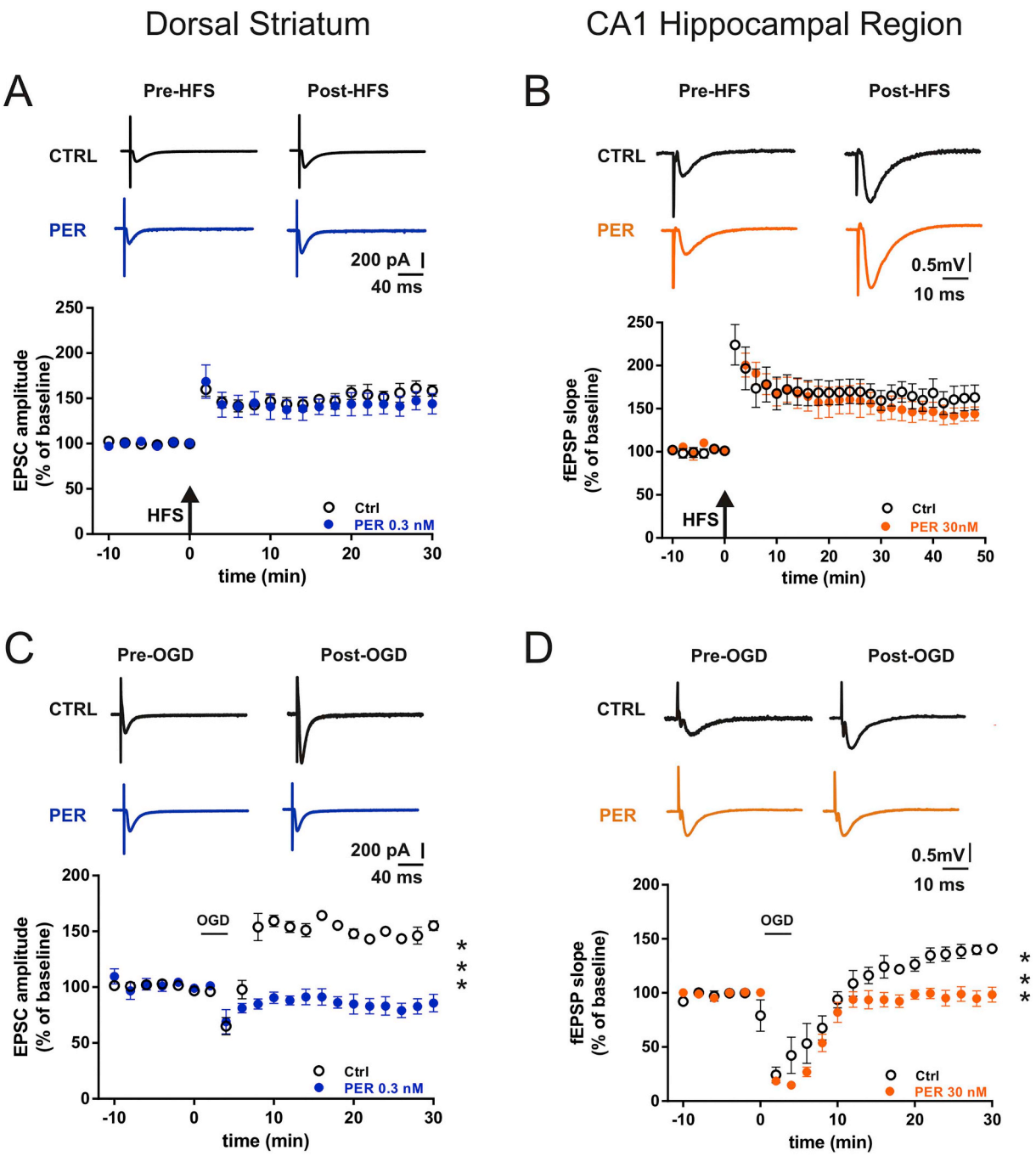

Fig. 4. Effect of PER on LTP and iLTP in the dorsolateral striatum and in CA1 hippocampal region. A, C) Representative traces of SPNsEPSCs recorded before and after the HFS (A) or the OGD protocol (C), in control conditions and in presence of 0.3 nM PER. The time-courses show the EPSCs amplitude before and after the HFS (A) or OGD (C) protocol, in control conditions (open circle) and in presence of 0.3 nM PER (blue circle). B, D) Representative traces of hippocampal fEPSPs recorded before and after the HFS (B) or the OGD (D) protocol, in control conditions and in presence of 30 nM PER (orange circle). Time-course of the fEPSPs amplitude before and after the HFS (B) or OGD (D) protocol, in control conditions (open circle) and in presence of 30 nM PER (orange circle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is worth noting that the neuroprotective effect of PER was achieved at very low concentrations with respect to those required for antiepileptic action (Biton, 2007; Costa et al., 2011). Similarly, the AEDs Zonisamide also showed neuroprotective effects against iLTP at doses lower than those used for anticonvulsant therapy (Costa et al., 2011).

A possible explanation to this neuroprotective effect could rely on the changes of AMPARs subunit composition induced by ischemic insult (Dos-Anjos et al., 2009), influencing the synaptic susceptibility to PER. Indeed, we found that OGD selectively increased theAMPARs expression of the GluA1 subunit, which possibly leads to the presence at synapses of novel GluA2-lacking Ca^{2+} AMPARs. PER seems to act specifically on GluA1 subunit, thus it might be possible that it is able to protect neurons at doses lower than those affecting synaptic transmission. This might be due to the high expression of GluA1 subunit after OGD in contrast to physiological conditions, in which the high majority of AMPARs express a GluA1/GluA2 or GluA2/GluA3 configuration. Indeed, PER neuroprotective effect might be related to the selective block of GluA1 subunit, rescuing physiological Ca^{2+}-impermeable AMPARs at synaptic level after an ischemic insult and preventing the

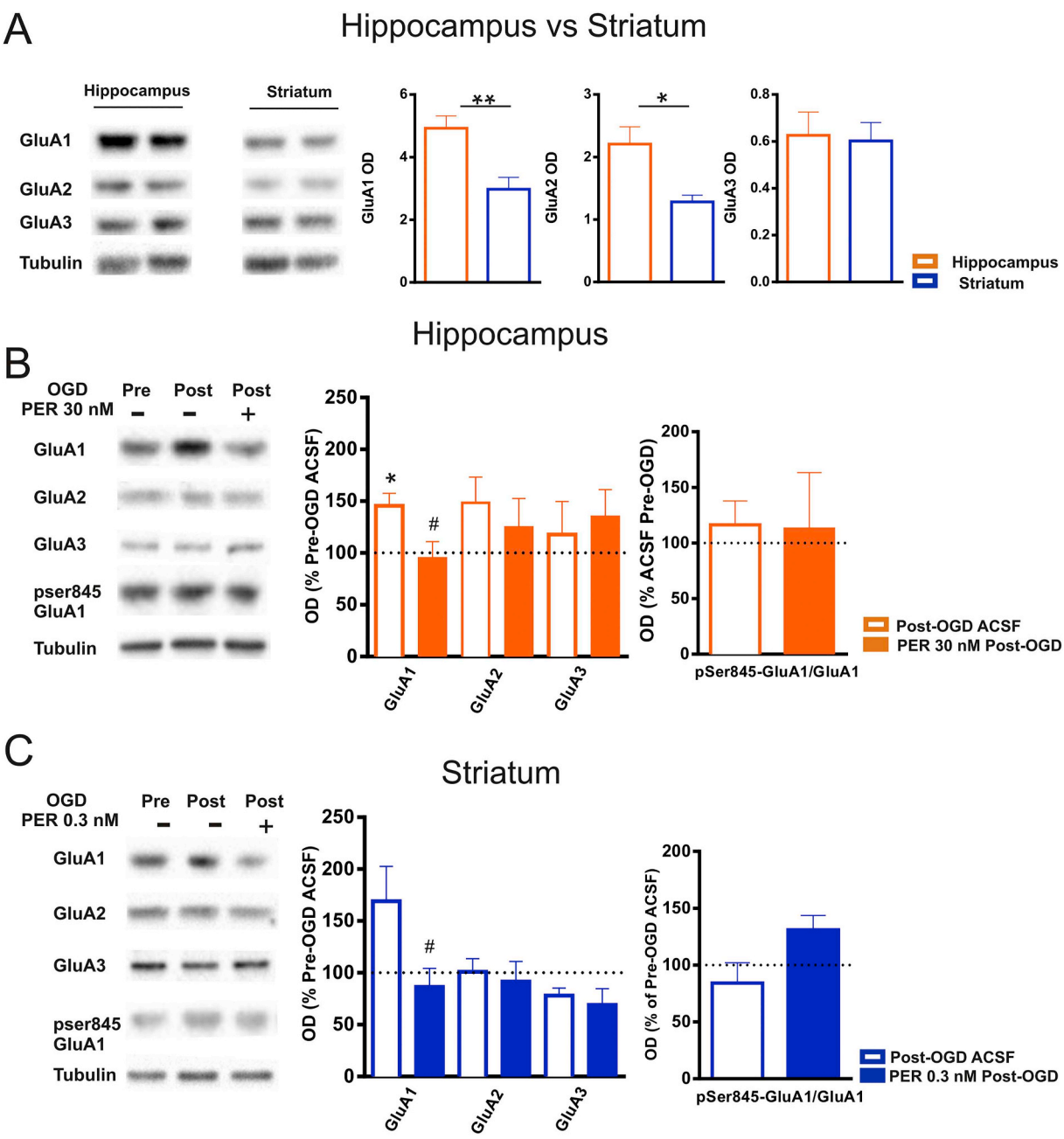

Fig. 5. Levels of AMPA receptor subunits in hippocampus and striatum in physiological conditions, after OGD insult and in presence of PER. A) WB analysis for GluA1, GluA2 and GluA3 subunits of the AMPARs in the homogenate fraction from striatum and hippocampus. Data are normalized against tubulin and are shown in the graph as mean ± SEM. **p < .01, *p < .05. B–C) WB analysis for GluA1, GluA1-p845, GluA2 and GluA3 subunits of the AMPARs in the triton insoluble postsynaptic fraction (TIF) from control and OGD-treated corticostriatal and hippocampal slices in absence or presence of PER. Data are normalized against tubulin and are shown in the graph as mean ± SEM. *p < .05.

harmful increase of intracellular Ca^{2+} leading to neuronal death. Moreover, the observation that PER-dependent neuroprotective effects were achieved at doses not significantly influencing basal excitatory transmission could also suggest that the PER-dependent beneficial effect acted through non-synaptic AMPA receptors or, more intriguingly, additionally unveiled neuroprotective pathways.

Interestingly, in support of the neuroprotective use of PER, it has been demonstrated that this drug was able to ameliorate the post-stroke motor function and the spatial working memory in a rat model of transient middle cerebral artery occlusion (MCAO), through possible anti-inflammatory and antioxidant mechanisms (Nakajima et al., 2018). Moreover, PER has been reported to reduce brain oedema, brain infarct volume and neuronal apoptosis following focal cerebral ischemia in rats (Niu et al., 2018). Unlike other compounds affecting AMPAR function, such as NBQX or Talampanel, which exhibit many shortcomings (Hanada et al., 2011; Langan et al., 2003), PER has a good oral bioavailability and favourable pharmacokinetic properties (Rogawski and Hanada, 2013), making it a good candidate for post-stroke neuroprotective strategy.

Although many AEDs, such as PER, showed potential neuroprotective effect, they are known to exert negative dose-dependent effects on cognitive functions (Helmstaedter and Witt, 2013; Witt et al., 2015). In fact, they have been shown to affect cognition in adult human subjects (Sgobio et al., 2010), morphogenesis and brain development by influencing synaptogenesis, synaptic plasticity and neural network formation (Caccamo et al., 2016; Ikonomidou and Turski, 2010).

Accordingly, Phenytoin can induce brain atrophy in patients after long-term use (Ney et al., 1994), while Valproic acid may produce morphologic alterations and impairment of specific hippocampal-dependent memory tasks (Sgobio et al., 2010). For this reason, the evaluation of the effects of AEDs on synaptic plasticity and cognition is critical.

Thus, we evaluated whether PER, at doses able to protect neurons from OGD, also affected the physiological expression of LTP in hippocampal and striatal areas. Our results suggest that the dose of PER exerting a neuroprotective effect against in vitro ischemia does not alter LTP of striatal neurons. In CA1 hippocampal region, while 30 nM PER is neuroprotective against OGD and LTP is spared, the neuroprotective effect of higher PER doses (100 nM) is associated to impaired LTP. Thus, it is conceivable that PER has a dose-dependent effect on LTP, reflecting its direct effect on synaptic transmission. These preclinical results could justify the clinical data of the literature, where Witt and colleagues demonstrated a considerable adverse effect of a higher drug load on cognition, especially on executive functions (Witt et al., 2015). For these reasons, further investigation to assess the effects of different doses of PER in vivo during experimental brain ischemia is required. Our results identified a possible therapeutic window allowing neuroprotection without impairing synaptic plasticity in distinct key brain areas involved in cognition. An in vivo investigation could define the proper PER administration protocol able to balance the beneficial and detrimental effects of PER on the whole brain.

5. Conclusion

Our results suggest that PER exerts a neuroprotective effect against energy deprivation in two brain regions particularly sensitive to ischemic insult and of fundamental importance in memory processes. A significant protection was reached at concentrations of the drug unable to affect synaptic transmission and plasticity, with different efficacy in striatum and hippocampus. Taken together, these findings support PER, a third generation AED, as a new candidate for further in vivo investigation for the treatment of brain ischemia.

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