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Erythropoietin induces homeostatic plasticity at hippocampal synapses

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Running title: Erythropoietin tuning of hippocampal synapses

29

Abstract

The cytokine erythropoietin (EPO) is the master regulator of erythropoiesis. Intriguingly, many studies have shown that the cognitive performance of patients receiving EPO for its hematopoietic effects is enhanced, which prompted the growing interest in the use of EPO-based strategies to treat neuropsychiatric disorders. EPO plays key roles in brain development and maturation, but also modulates synaptic transmission. However, the mechanisms underlying the latter have remained elusive. Here, we show that acute (40-60 min) exposure to EPO presynaptically downregulates spontaneous and afferent-evoked excitatory transmission, without affecting basal firing of action potentials. Conversely, prolonged (3h) exposure to EPO, if followed by a recovery period (1h), is able to elicit a homeostatic increase in excitatory spontaneous, but not in evoked, synaptic transmission. These data lend support to the emerging view that segregated pathways underlie spontaneous and evoked neurotransmitter release. Furthermore, we show that prolonged exposure to EPO facilitates a form of hippocampal long-term potentiation (LTP) that requires non-canonical recruitment of calcium-permeable AMPA receptors (CP-AMPARs) for its maintenance. These findings provide important new insight into the mechanisms by which EPO enhances neuronal function, learning and memory.

Introduction

Erythropoietin (EPO) is a pleiotropic hormone from the type I cytokine super-family best known as a crucial regulator of erythropoiesis and tissue oxygenation homeostasis. In addition, EPO signaling has been shown to play key roles in the central nervous system (CNS), where neurons and astrocytes express a specific non-hematopoietic EPO receptor (**Fond et al., 2012**). EPO is an important regulator of neuronal proliferation and migration during CNS development and also following injury to the mature brain, such as during post-stroke recovery (**Tsai et al., 2006**). Intriguingly, in addition to its role in neurogenesis, early studies reported positive cognitive effects in patients regularly receiving EPO for chronic kidney disease (**Kambova, 1998; Pickett et al., 1999**). Similar pro-cognitive effects have since been reported in patients with schizophrenia (**Ehrenreich et al., 2007**), bipolar disorder (**Miskowiak et al., 2014**) and type 1 diabetes (**Kristensen et al., 2013**).

These cognitive-enhancing effects might be partially explained by the fact that EPO raises hemoglobin levels and improves brain oxygenation or could reflect the anti-apoptotic, anti-oxidative and anti-inflammatory properties of this cytokine (**Brines and Cerami, 2005; Sirén, 2009; Fond et al., 2012**). However, treatment with EPO has been shown to amplify the expression of pro-plasticity genes in an experimental stroke model at early time points, even before EPO would be expected to reduce inflammation or neuronal apoptosis (**Mengozzi et al., 2012**).

In keeping with these data, recent evidence indicates that EPO directly modulates neuronal activity and plasticity (**Adamcio et al., 2008**; **Kamal et al., 2011**; **Sargin et al., 2011**). However, differences in the experimental models used to investigate EPO signaling have yielded some inconsistencies in results (**Supplementary Table 1**). We propose that, at least partially, this is due to the fact that differences in effect between

78   acute neuronal tuning *vs.* network effects of prolonged exposure have not been

79   accounted for.

80   Here, we directly compared the effects of acute EPO application (aEPO) with those of a

81   more prolonged exposure (pEPO) using *ex vivo* and *in vitro* experimental models that

82   isolate the actions of EPO to the neuronal/astrocytic compartment. Our results

83   demonstrate that acute EPO acts as an inhibitor of synaptic transmission,

84   downregulating excitatory and upregulating inhibitory spontaneous activity. In contrast,

85   more prolonged exposure (3h) to EPO, followed by a recovery period (1h), resulted in

86   opposite, homeostatic changes in spontaneous synaptic transmission. These results

87   resemble those observed following exposure to an activity deprivation (AD) paradigm

88   with tetrodotoxin (TTX, 1 μM), a sodium channel blocker, and APV (50 μM), a N-

89   methyl-D-aspartate receptor (NMDAR) antagonist (**Sutton et al., 2006**; **Félix-Oliveira**

90   **et al., 2014**). Therefore, in order to better understand the consequences of EPO's

91   homeostatic actions, we directly compared the effects of prolonged EPO against those

92   of the AD protocol on network excitability and hebbian synaptic potentiation.

93

Materials and Methods

Animals

All experiments were conducted using Wistar or Sprague-Dawley rats, as indicated. Animals were kept under standardized temperature, humidity and lighting conditions, with access to water and food *ad libitum*. All animal procedures were carried out according to the Portuguese law and the European Community Guidelines for Animal Care (European Union Council Directive – 2010/63/ EU). Throughout the underlying experimental work, care was taken to minimize the number of animals sacrificed.

Primary Hippocampal Cultures

Pregnant Sprague-Dawley rats were obtained from Harlan Interfauna Iberia (Barcelona, Spain). Animals were deeply anesthetized with halothane (2-Bromo-2-Chloro-1,1,1Trifluoroethane) in an anesthesia chamber before being sacrificed by decapitation. Briefly, embryos (E17-E18) were rapidly removed from the uterus, their brains removed and placed in cold Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution supplemented with 0.37% glucose (HBSS-glucose). After brain isolation, meninges were gently removed from the hemispheres. All hippocampi were collected in fresh HBSS-glucose and trypsinization was carried out (0,350 ml of 2,5% trypsin) at 37 ºC for 15 min in a water bath. Afterwards, trypsin solution was gently removed and 30% Fetal Bovine Serum (FBS) in HBSS-glucose was added and let stand for 5 min at room temperature (RT, 22-24ºC) to quench trypsin activity. The pellet was resuspended in Neurobasal medium (Neurobasal-B27: Neurobasal supplemented with 0.5 mM glutamine, 2% B27, 25 U/mL penicillin/streptomycin) and 25 mM glutamic acid. Cell suspension was filtered using a nylon filter (Cell Strainer 70 µM, BD Falcon™) and cell density was

119   determined by counting cells in a 0.4% trypan blue solution using a hemacytometer.

120   Cells were plated at 4×10^4 cells/cm^2 in 24-well plates with glass coverslips (Marienfeld,

121   Germany) coated with poly-D-lysine (PDL, 50 µg/ml). The cultures were maintained

122   for a maximum of 21 days, in an incubator with a humidified 37ºC and 5% CO_2

123   atmosphere with no media exchange.

124

125   *Acute Slice preparation and incubation*

126   Young Wistar rats (3-5 weeks old) were killed by decapitation under deep isoflurane

127   anesthesia. The brain was quickly removed, hemisected and both hippocampi were

128   dissected free within ice-cold dissecting solution containing (in mM): 110 sucrose; 2.5

129   KCl; 0.5 $CaCl_2$; 7 $MgCl_2$; 25 $NaHCO_3$; 1.25 NaH_2PO_4; and 7 glucose; previously

130   gassed with 95% O_2 and 5% CO_2, pH 7.4.

131   For extracellular recordings, slices (350 µm-thick) were cut perpendicularly to the long

132   axis of the hippocampus with a McIlwain tissue chopper (unless stated otherwise) and

133   allowed to recover functionally and energetically for at least 60 min at RT, in a resting

134   chamber filled with artificial cerebrospinal fluid (aCSF) (containing, in mM: 124 NaCl;

135   3 KCl; 1.25 NaH_2PO_4; 26 $NaHCO_3$; 1 $MgSO_4$; 2 $CaCl_2$; and 10 glucose), previously

136   gassed with 95% O_2 and 5% CO_2, pH 7.4.

137   For patch-clamp recordings, acute hippocampal (300 µm-thick) slices were cut using a

138   vibratome (VT1000 S; Leica, Nussloch, Germany) in the aforementioned ice-cold

139   dissecting solution. Slices were first incubated for 30 min at 35°C in gassed aCSF

140   followed by at least 60 min of recovery at RT.

141   Hippocampal slices were incubated with vehicle, with EPO (2.4 IU/ml) alone, with both

142   TTX (1 µM) and AP-V (50 µM) or with these three drugs simultaneously, for 3 hours,

143   at RT, in gassed aCSF. For some experimental designs, this was followed by a

144   washout/recovery period of 1 hour in gassed aCSF at RT, as indicated. EPO

145   concentration (2.4 IU/ml) corresponds to a concentration 8-times (2^3) higher than that

146   used by others in cultured cells (**Adamcio et al., 2008**). This was done since it is more

147   difficult for EPO to permeate hippocampal slices (300-350 µm-thick), than cultured

148   cells (in monolayer).

149

150   *Electrophysiology*

151   *Extracellular ex vivo Recordings.*

152   Slices were transferred to a submerging chamber (1 ml) and continuously superfused at

153   a 3 ml/min rate with gassed aCSF at 32ºC. Evoked orthodromic field excitatory

154   postsynaptic potentials (fEPSP) were recorded extracellularly through a microelectrode

155   filled with 4 M NaCl (2–8 MΩ resistance) placed in the stratum radiatum of the CA1

156   area, as previously described (see e.g. **Rodrigues et al., 2014**). One pathway of the

157   Schaffer collateral (SC)/commissural fibers was stimulated (rectangular pulses of 0.1

158   ms duration) at every 15 s (for I/O curves, basal synaptic transmission and PPF

159   recordings) or every 20 s (for LTP recordings), by a bipolar concentric wire electrode

160   placed on the Schaffer fibers in the stratum radiatum, in the CA1 area. The initial

161   intensity of the stimulus was adjusted to obtain a submaximal fEPSP slope with a

162   minimum population spike contamination, near one-fourth of the fEPSP slope obtained

163   with supramaximal stimulation. The averages of eight (for I/O curves, basal synaptic

164   transmission and PPF recordings) or six consecutive fEPSP (for synaptic plasticity

165   recordings) were obtained and the slope of the initial phase of the potential was

166   quantified. Recordings were obtained with an Axoclamp 2B amplifier (Axon

167   Instruments, Foster City, CA), digitized and continuously stored on a personal computer

168   with the LTP program (**Anderson and Collingridge, 2001**). All the protocols detailed

169   below were started only after a stable baseline of at least 20 minutes. In the tracings

170   shown, the stimulus artifact was truncated for clarity.

171   *Basal Synaptic Transmission.* Alterations in synaptic transmission induced by EPO (2.4

172   IU/ml) were evaluated as % change in the average slope of the fEPSP 50-60 min after

173   EPO application, relatively to the average slope of the fEPSP measured during the 10

174   min that preceded addition of EPO to the superfusing solution.

175   *Paired-Pulse Facilitation (PPF) Recordings*. PPF was quantified as the percentage of

176   slope facilitation between two consecutive fEPSPs (fEPSP2/fEPSP1) elicited with a 50-

177   ms interstimulus interval. PPFs were continuously elicited for 60 min; EPO was added

178   to the superfusing solution 20 min after having started the PPF protocol. Alterations in

179   PPF efficiency induced by EPO (2.4 IU/ml) were evaluated as % change in the average

180   PPF ratio 30-40 min after EPO application, relatively to the average PPF ratio measured

181   during the 10 min that preceded the addition of EPO to the superfusing solution. As a

182   control, in different slices, PPFs were continuously elicited for 60 min, adding only

183   vehicle to the superfusing solution, to test whether continuously eliciting PPFs for that

184   time period would *per se* change PPF efficiency.

185   *Input/Output Curves*. The stimulus delivered to the slice was decreased until no fEPSP

186   was evoked and subsequently increased in 20 μA steps. Data from three consecutive

187   averages of eight fEPSPs were collected for each stimulation intensity. Inputs delivered

188   to slices typically ranged from 80 μA to a supramaximal stimulation of 320 μA. fEPSP

189   slope was plotted as a function of stimulus intensity. The maximum slope values were

190   obtained by extrapolation upon nonlinear fitting of the I/O curve and an F test was used

191   to determine differences between the parameters. Whenever the stimulus was intense

192   enough to elicit a population spike (depolarization that follows the fEPSP), its

193   amplitude was measured. Each popspike amplitude replicate was plotted as a function

194 of the slope of the associated fEPSP and the popspike/fEPSP ratio calculated as the

195 quotient between these values.

196 *LTP induction.* A weak LTP-inducing θ-burst protocol consisting of four trains of 100

197 Hz, four stimuli, separated by 200 ms was used (1x4x4). The intensity of the stimulus

198 was never changed throughout the experiment. LTP magnitude was quantified as the %

199 change in the average of the fEPSP taken from 50 to 60 min after LTP induction

200 relative to the average slope of the fEPSP measured during the 10 min that preceded

201 induction of LTP. Post-tetanic potentiation (PTP) was assessed as the average fEPSP

202 slope obtained in the first 8 min after LTP induction (**Habets and Borst, 2007**).

203 For the experiments in **Figure 7**, stimulation was delivered, every 10 s, alternatively to

204 two independent pathways of the Schaffer collateral/commissural fibers. In each

205 individual experiment, the LTP-inducing paradigm was delivered to one of the

206 pathways and, 1 hour thereafter, to the other pathway; each pathway was used as control

207 or test in alternate days. NASPM (20 μM) was added to the superfusing solution 20 min

208 after the induction of the 2nd LTP. This protocol allowed to test the effect of NASPM on

209 LTP maintenance, with the first LTP serving as an internal control. The LTP inducing

210 protocol used was the same and LTP magnitude was quantified as explained above.

211 *Antidromic Population Spike (Popspike) Recordings.* Hippocampal slices used were

212 prepared as described above for patch-clamp recordings. Evoked antidromic popspikes

213 were recorded extracellularly through a microelectrode filled with 4 M NaCl (2–8 MΩ

214 resistance) placed in CA1 pyramidal cell layer. Antidromic responses were evoked by

215 stimulation in the alveus, every 15 s, by a bipolar concentric wire electrode (**Kabakov**

216 **et al., 2012**). The initial intensity of the stimulus was adjusted to obtain a submaximal

217 popspike amplitude with minimal contamination. Averages of eight responses were

218 obtained and the popspike amplitude was calculated as the amplitude from the popspike

peak to the intersection with an interpolated tangent line drawn between the pre-popspike peak and the post-popspike peak (**Figure S1A**). Recordings were obtained with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), digitized and continuously stored on a personal computer with the LTP program (**Anderson and Collingridge, 2001**). In order to demonstrate that nonsynaptic excitatory transmission underlies the popspikes we recorded, control experiments were performed (for a representative experiment, see **Figure S1B**). Alterations in popspike amplitude induced by EPO (2.4 IU/ml) were evaluated as % change in the average amplitude of the popspike 50-60 min after EPO application, relatively to the average amplitude of the popspike measured during the 10 min that preceded addition of EPO to the superfusing solution.

Patch-clamp Recordings.

Whole cell patch-clamp recordings were obtained either from hippocampal CA1 pyramidal cells or from hippocampal cultured neurons, as indicated. These were visualized with an upright microscope (Zeiss Axioskop 2FS) equipped with infrared video microscopy and differential interference contrast optics. Recordings were performed at RT in current-clamp or voltage-clamp mode with an Axopatch 200B (Axon Instruments) amplifier. For current-clamp recordings and for miniature or spontaneous excitatory postsynaptic current (mEPSC or sEPSC, respectively) recordings, patch pipettes (4- to 7-MΩ resistance) were filled with an internal solution containing (in mM): 125 K-gluconate, 11 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine, pH 7.3, adjusted with 1 M KOH, 280–290 mOsm. For miniature inhibitory postsynaptic current (mIPSC) recordings and AMPA- and NMDA-mediated responses, the internal solution contained

244 (in mM): 125 CsCl, 8 NaCl, 1 CaCl$_2$, 10 EGTA, 10 HEPES, 10 glucose, 5 MgATP, and

245 0.4 NaGTP, pH 7.2, adjusted with CsOH (50 wt% in H$_2$O), 280–290 mOsm. Acquired

246 signals were filtered using an in-built, 2-kHz, three-pole Bessel filter, and data were

247 digitized at 5 or 10 kHz under control of the pCLAMP 10 (Molecular Devices) software

248 program. The junction potential was not compensated for, and offset potentials were

249 nulled before giga-seal formation. Small voltage steps (–5 mV, 50 ms) were used to

250 monitor the access resistance throughout experiments. The holding current was also

251 constantly monitored and experiments in which any of these parameters varied by more

252 than 20% were discarded.

253 *Current-clamp recordings*. The resting membrane potential was measured immediately

254 upon establishing whole cell configuration. Changes in membrane potential and action

255 potentials were evoked under current-clamp mode by injection of 500 ms current pulses

256 (25 to 275 pA in 25 pA increments) from an initial holding potential (Vh) of –70 mV.

257 The threshold for action potential (AP) generation was determined as the difference

258 between the resting membrane potential (V_m) and the membrane potential at which

259 phase plot slope reached 10 mV/ms (V_t) (**Naundorf et al., 2005**). When studying the

260 effect of acute EPO/vehicle superfusion upon spiking frequency, neurons were initially

261 current-clamped to –60 mV (the closest to our sample average resting membrane

262 potential) and this holding current was kept constant throughout the experiment. After

263 10 min of baseline recording, EPO (2.4 IU/ml) or vehicle were added to the superfusion

264 solution and membrane potential variations were recorded for at least 40 min. The

265 average spiking frequency was calculated as the frequency of APs within the entire

266 period of superfusion with EPO/vehicle.

267 *Spontaneous activity*. Spontaneous mEPSCs were recorded in aCSF supplemented with

268 TTX (500 nM) and gabazine (2 μM). Spontaneous mIPSCs were recorded in aCSF

269   supplemented with TTX (500 nM) and kynurenic acid (1 mM). For sEPSC recordings

270   only gabazine (2 µM) was added to perfusion. Analysis of spontaneous events was

271   performed using the Synaptosoft Mini Analysis Program software with the amplitude

272   threshold for event detection set at 3x the average root-mean-square noise.

273   *Synaptic Failure Rate*. These experiments were performed using minimum stimulation.

274   Recordings were performed in the continuous presence of picrotoxin (100 µM).

275   Synaptic responses were obtained with a bipolar electrode placed over Schaffer

276   collateral fibers and stimuli (100 µs) were delivered every 5 s. AMPA-mediated

277   responses were measured as the peak amplitude value of the EPSC at -60 mV and

278   NMDA-mediated responses as the peak amplitude value of the EPSC at $+40$ mV, at a

279   latency where AMPA responses had fully decayed (30-60 ms) (**Arendt et al., 2013**).

280   The criterion used for defining success of synaptic transmission was peak amplitude

281   values greater than 20 pA for AMPA-mediated responses and 10 pA for NMDA-

282   mediated responses. Failure rate was calculated as a percentage of failed evoked

283   responses over 50 sweeps. In the amplitude scatter plots, for ease of visualization, the

284   amplitudes of failure trials were plotted as zero. To facilitate the interpretation of the

285   tracings shown, the stimulus artifact was blanked.

286

287   *Live Imaging of SEP-GluA2*

288   Live imaging experiments were performed using primary cultured hippocampal neurons

289   at DIC 17-24, prepared as routinely in the lab (**Martin and Henley, 2004**), 12h-24h

290   upon infection with Sindbis virus carrying Super Ecliptic Phluorin-GluA2 (SEP-GluA2)

291   (**Ashby et al., 2004; 2006**). Neurons were placed on a heated stage (set at 35ºC) of a

292   Nikon TE-2000E inverted microscope (Garden City, NY) and were continually perfused

293   at 3 ml/min with warm Earle's solution containing either TTX (500 nM) alone for the

294 baseline and washout period or TTX (500 nM) and EPO (2.4 IU/mL) during the test

295 period. Low pH external solutions contained equimolar MES instead of HEPES (pH

296 adjusted to 6). NH_4Cl (50 mM) was used in place of equimolar NaCl to collapse pH

297 gradients. Fluorescence was excited using a 63x water-immersion objective by a 488

298 nm laser light and emission was detected through a 505 nm long pass filter. Time series

299 were collected as repetitively scanned images. All SEP-GluA2 experiments included a

300 brief (30 sec) low pH wash at the end of each experiment, to subtract the remaining

301 signal (internal AMPAR fluorescence) from the total and thus obtain the signal

302 corresponding to surface-expressed AMPARs.

303

304 *Immunocytochemistry and Confocal Microscopy*

305 DIC 15 and DIC 21 hippocampal neurons were washed with Phosphate Buffered Saline

306 (PBS) (containing in mM: 137 NaCl, 2.1 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4.2H_2O$, pH

307 7.40), containing sucrose whenever needed to correct the solution to the medium

308 osmolarity. Cells were then fixed with 4% phosphate buffered paraformaldehyde (PFA)

309 at RT, for 20 minutes. Excess of PFA was removed by washing with PBS solution.

310 Cells were permeabilized and blocked for 1h using PBS containing 1% bovine serum

311 albumin (BSA, Sigma-Aldrich) and 0.1% Tween 20, at RT, and, then, incubated for 1

312 hour, at RT, with a mouse monoclonal primary antibody anti-MAP2 (1:200) and a

313 rabbit polyclonal primary antibody anti-EpoR (1:200; dilution range typically used

314 varies from 1:100 to 1:500, **Bernaudin et al., 1999**; **Sanchez et al., 2009**), in blocking

315 solution. Washes to remove the excess of primary antibody were then performed in

316 PBS. Hippocampal cultured neurons were subsequently incubated for 2 hours, at RT,

317 with a donkey anti-mouse and donkey anti-rabbit secondary antibodies respectively

318 conjugated to the fluorescent label Alexa Fluor 568 (1:500) and 488 (1:250) in blocking

319   solution. Images of neurons were captured using a confocal point-scanning microscope

320   (Zeiss LSM 710, Germany) with a 63x objective and using the software ZEN 2009

321   (Carl Zeiss MicroImaging). The pixel size in the object space was 0.13 μm and the

322   captured images were 1024 x 1024 pixels size. Images were stored and analyzed in an

323   uncompressed 16-bit .tiff format.

324   As a quality control of the anti-EpoR antibody, a blocking peptide competition assay

325   was also performed (**Figure S2**). In this case, DIC 15 hippocampal neurons were either

326   incubated with the primary antibody or with the primary antibody after being pre-

327   incubated with the blocking peptide in a proportion of either 1:5 or 1:10 (primary

328   antibody:blocking peptide) (**Brownjohn and Ashton 2014**).

329   *EpoR protein intensity quantification*. Hippocampal neuronal images with a 16-bit .tiff

330   format were analyzed by using ImageJ (1.46r, NIH) software. EpoR expression in the

331   soma and dendrites was evaluated by quantifying respectively the mean gray value of

332   the soma and the mean gray value of the dendrites (by definition, the mean gray value

333   corresponds to the sum of the gray values of all pixels in the selected area divided by

334   the number of pixels). First, the threshold of the whole neuronal area was adjusted on

335   the MAP2 channel, being this region selected as the region of interest (ROI) for the

336   neuron (A). The cell body was selected by drawing a polygon (B) and the whole image

337   was also selected (C). These three ROIs were then combined in order to limit the areas

338   to the cell body (A∩B), the dendrites (A\B) and the background (C\A). By measuring,

339   the program gave the mean gray values of the EpoR channel for the three combined

340   ROIs plus the background. Then, the background mean gray value was subtracted from

341   the mean gray values of the cell body and dendrites.

Drugs

343   EPO was purchased from Jena Bioscience (Jena, Germany) and was prepared in

344   distilled water with 0.1% BSA as 100 µg/mL stock solution. TTX (tetrodotoxin citrate),

345   gabazine, DL-APV (DL-2-amino-5-phosphonopentanoic acid), kynurenic acid and

346   CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) were all purchased from Abcam

347   Biochemicals (Cambridge, MA, USA) and were prepared in distilled water as 1, 10, 50,

348   100 and 10 mM stock solutions, respectively. Picrotoxin was also purchased from

349   Abcam Biochemicals (Cambridge, MA, USA) and prepared in dimethylsulfoxide

350   (DMSO) as a 50 mM stock solution. K252a was purchased from Tocris Cookson

351   (Ballwin, MO, USA) and made up in a 1 mM stock solution in DMSO. NASPM (1-

352   naphthylacetylspermine) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and

353   prepared as 10 mM stock solution in distilled water. For the live imaging assays, EPO,

354   TTX and APV from the same batches as those used for the electrophysiology

355   experiments were used. For the immunocytochemistry procedures, to identify EpoR, a

356   polyclonal rabbit antibody [Santa-Cruz Biotechnology, No. sc-697 (M-20), stock

357   concentration 200 µg/mL] raised against amino acids 488-507, mapping at the C-

358   terminal cytoplasmic domain (amino acids 273-507) of the precursor form of mouse

359   EpoR, was used. As dendritic marker, we used a mouse anti-MAP2 monoclonal

360   antibody (Chemicon, Millipore, No. MAB3418, stock concentration 1 mg/mL). The

361   reagents used for primary hippocampal cultures were purchased from Invitrogen

362   (Carlsbed, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), unless stated

363   otherwise.

364

365

366

367 *Statistics*

368 All data were tested for normality with Kolmogorov-Smirnov test. Data presented as

369 mean ± s.e.m have normal distributions and t-test or one-way ANOVA were used to test

370 for significance. Otherwise, data were shown as median and quartiles and Mann-

371 Whitney test was used instead.

Results

EPO acutely (aEPO) downregulates evoked transmission by operating presynaptic mechanisms

We first reexamined the effect of acute EPO (aEPO) superfusion on afferent-evoked transmission to CA1 neurons, by eliciting orthodromic fEPSPs in hippocampal slices. We found that aEPO significantly inhibited fEPSP slope (**Figure 1B1**) even when applied at a lower, more physiological concentration (2.4 IU/ml) than previously used (50 IU/ml in **Kamal et al., 2011**). EPO signals through EPO receptors (EpoRs), which are frequently found as homodimeric tyrosine kinase receptors (RTKs) in the CNS (**Digicaylioglu and Lipton, 2001**; **Ma et al., 2016**). As shown in **Figure 1B2-C,** the effect of EPO was prevented by the tyrosine kinase inhibitor, k252a (200 nM), thus suggesting that EPO downregulates afferent-evoked transmission at SC-CA1 synapses through homodimeric EpoRs. Interestingly, we found a consistent time lag of approximately 10 minutes before aEPO's effect became apparent (**Figure 1B1**). This lag was described before by **Kamal et al., 2011** and may relate to the fact that none of the known signaling molecules downstream to the EpoR are able to directly modulate membrane ionic currents (**Ma et al., 2016**); thus, additional steps (e.g., ion channel membrane trafficking or degradation) may be required.

In order to further dissect the effect of acutely applied EPO on activity-dependent synaptic transmission, namely to determine whether it should be ascribed to pre- and/or postsynaptic mechanisms, we performed a paired-pulse facilitation (PPF) protocol, evoked antidromic responses from CA1 neurons and recorded spontaneous changes in membrane potential intracellularly from CA1 pyramidal cells (PCs) soma. We found that (1) the paired-pulse facilitation (PPF) ratio was significantly increased after aEPO

397   (40 min, 2.4 IU/ml) perfusion (n=5, *p*=0.0019, paired t-test; **Figure 1D**), which

398   indicates that EPO reduced the probability of excitatory neurotransmitter release; (2) the

399   popspike amplitude of antidromically-evoked responses was unaffected by aEPO (60

400   min, 2.4 IU/ml; n=5-6, *p*=0.8409, unpaired t-test; **Figure 1E-F**), which suggests that

401   EPO has no effect on non-synaptically driven excitatory activity of postsynaptic PCs;

402   and (3) the frequency of spontaneous action potentials (AP) recorded from the soma of

403   CA1 PCs, current-clamped to a near-resting membrane potential of −60 mV, was not

404   affected by aEPO (40 min, 2.4 IU/ml) [vehicle: 0.56 (0.26-1.58) Hz; EPO: 0.59 (0.01-

405   1.55) Hz; n= 6-9, *p*=0.7756, Mann-Whitney test; data represented as median

406   (interquartile range); **Figure 1G-H**]. Taken together, these data suggest that acute

407   EPO's effect on neuronal transmission is inherently synaptic and that EPO should

408   mainly operate through presynaptic mechanisms to inhibit transmission at SC-CA1

409   synapses.

410   Consistent with these results, live-imaging assays of GFP-tagged postsynaptic GluA2

411   AMPA receptor (AMPAR) subunits showed that neither the punctate (synaptic

412   AMPARs) nor the diffuse surface signal (dendritic/perisynaptic AMPARs) were

413   significantly affected by aEPO superfusion (**Figure 2A**). Such lack of effect on

414   postsynaptic AMPAR trafficking could hypothetically be attributed to a lack of

415   expression of EpoR at neuronal dendrites. Immunocytochemistry studies on mature

416   cultured hippocampal neurons showed that the EpoR mainly localized to the cell soma,

417   but was also expressed, at lower levels, in the dendritic compartment (**Figure 2B**).

418   Hence, a lack of expression of the EpoR should not account for the absence of effect on

419   AMPAR trafficking.

420

421   *Prolonged exposure to EPO (pEPO) drives the homeostatic upscaling of excitatory and*

422   *the downscaling of inhibitory spontaneous event, only if followed by a recovery period*

423   The results reported so far suggest that EPO acutely acts to inhibit synaptic

424   transmission, mainly through presynaptic mechanisms. Next, we aimed to test whether a

425   more prolonged exposure to EPO's synaptic manipulation would be able to produce

426   enduring, and perhaps more physiologically relevant, changes in synaptic transmission.

427   Homeostatic forms of synaptic plasticity ultimately ensure long-term stability of

428   neuronal function, by scaling synaptic-receptor currents up or down after prolonged

429   activity decrease or increase, respectively (e.g., **Turrigiano et al., 1998**). Such synaptic

430   scaling is usually measured as a change in quantal or miniature synaptic transmission

431   ('minis'), which accounts for the AP-independent fusion of synaptic vesicles at

432   presynaptic terminals (e.g., **Sutton et al., 2006**; reviewed by **Pozo and Goda, 2010**).

433   Therefore, we next investigated the effects of EPO on spontaneous miniature events.

434   First, we tested the effect of aEPO (2.4 IU/ml; **Figure 3C**), which significantly

435   decreased the frequency of miniature excitatory postsynaptic currents (mEPSCs)

436   (baseline:0.42±0.10 Hz; after 40 min:0.26±0.08 Hz; n=7, *p*=0.0304, paired t-test;

437   **Figure 3D**) and significantly increased the frequency of miniature inhibitory

438   postsynaptic currents (mIPSC) (baseline:3.55±0.90 Hz; after 40 min:4.10±1.00 Hz; n=5,

439   *p*=0.0088, paired t-test; **Figure 3E**). No significant effects were observed in

440   mEPSC/mIPSC amplitude (**Figure 3D-E**), consistent with our previous thesis that EPO

441   mainly operates through presynaptic mechanisms. Interestingly, these effects endured,

442   even after prolonged (3h) exposure to EPO (pEPO, 2.4 IU/ml), followed by continuous

443   EPO superfusion throughout the recording period (**Figure 3F**). Indeed, under these

444   conditions, mEPSC frequency was found to be decreased (veh:0.20±0.02 Hz;

445   EPO:0.12±0.02 Hz; n=5-8 per condition, *p*=0.0208, unpaired t-test; **Figure 3G**), and

446   mIPSC frequency was found to be increased (veh:1.67±0.36 Hz; EPO:3.41±0.53 Hz;

447   n=9 per condition, p=0.0148, unpaired t-test; **Figure 3H**).

448   Since changes in the spontaneous-related vesicle pool suffice to elicit homeostatic

449   phenomena (**Reese and Kavalali, 2015**), we next tested if prolonged (3h) exposure to

450   EPO (pEPO), followed by a recovery period (1h) to allow unmasking of the acute

451   effects, could induce lasting homeostatic changes in neuronal transmission. Under these

452   conditions, CA1 pyramidal cells displayed a higher frequency of mEPSCs

453   (veh:0.08±0.01 Hz; EPO:0.29±0.08 Hz; n=11 per condition, p=0.0408, unpaired t-test

454   with Welch's correction; **Figure 3J**). Identical effects were observed when recording

455   from hippocampal cultured neurons (**Figure S3A**), suggesting that the homeostatic

456   changes induced by pEPO are inherently synaptic and do not require an intact circuitry.

457   Even though quantal glutamate release at central synapses is assumed to mainly arise

458   from AP-independent vesicle fusion, it can also result from AP-dependent activity (**Ye**

459   **et al., 2010**). For this reason, we addressed the effects of pEPO upon spontaneous

460   EPSCs (sEPSCs), which comprise responses to both types of release. No significant

461   differences in sEPSC amplitude or decay were observed, although there was a trend for

462   increased sEPSC frequency (**Figure S3B-C**). Since EPO does not affect spontaneous

463   AP generation (**Figure 1H**), it is plausible to assume that spontaneous AP-driven

464   events, if unaffected by EPO, might "blur" the scaling of co-recorded AP-independent

465   events.

466   GABAergic transmission was also affected by pEPO, when followed by a recovery

467   period, through a downscaling in mIPSC frequency (veh:1.72±0.24 Hz; EPO:1.06±0.14

468   Hz; n=6-7, p=0.0312, unpaired t-test; **Figure 3K**), which is reciprocal to the scaling

469   effect of EPO on mEPSCs.

470 In addition to adjusting synaptic weights, homeostatic plasticity can also promote silent

471 synapse formation (**Arendt et al., 2013**) or trigger changes in intrinsic membrane

472 properties and neuronal excitability (**Desai et al., 1999**), to maintain stability in AP

473 firing rates despite activity manipulation (**Hengen et al., 2013**; **Keck et al., 2013**). In

474 CA1 PCs exposed to pEPO (2.4 IU/ml, 3h), followed by a recovery period (1h), whole-

475 cell current-clamp recordings showed no significant differences in instantaneous AP

476 firing frequency in response to soma depolarization (top parameter of fitted curves±s.e.:

477 veh:34.89±1.50 Hz, EPO:37.68±2.27 Hz, p>0.05; slopes of fitted curves±s.e.:

478 veh:0.0096±0.0018 Hz/pA, EPO:0.0094±0.0024 Hz/pA; n=15 per condition, p>0.05,

479 sum-of-squares F-test; **Figure 4A-C**), nor in the maximum AP frequency, resting

480 membrane potential (RMP), AP firing threshold or delay to first AP (**Table 1**). Hence,

481 prolonged EPO does not seem to affect membrane excitability, nor the ability of the

482 postsynaptic neuron to compute information under conditions of strong depolarization.

483 Glutamatergic synapses that lack AMPARs and only contain NMDARs are functionally

484 silent despite intact presynaptic glutamate release, due to the voltage-dependent

485 magnesium blockade of NMDARs (**Isaac et al., 1995**). To detect silent synapses,

486 voltage-clamped recordings from CA1 pyramidal cells were performed, and excitatory

487 synaptic transmission was elicited by minimal electrical stimulation, which produced

488 failures in ~50% of trials (**Figure 4D**). Epochs of 50 trials were recorded at −60 mV and

489 +40 mV for each cell, and the failure rate at these two holding potentials was calculated.

490 The failure rate was comparable between the negative and the positive holding

491 potentials both in vehicle- (−60 mV:42.67±3.65%; +40 mV:37.33±7.48%; n=9,

492 p=0.4192, paired t-test) and in pEPO-treated slices (−60 mV:43.25±6.58%; +40

493 mV:46.75±5.10%; n=8, p=0.6033, paired t-test; **Figure 4E-G**) for 3h, followed by a

494 recovery period (1h). These data suggest that most SC-CA1 synapses are active in our

495 experimental conditions and that the number of silent synapses is not modified by

496 prolonged exposure to EPO.

497

498 *Prolonged application of EPO (pEPO) decreases basal afferent-evoked synaptic*

499 *transmission without modifying CA1 PC input-output function*

500 Thus far, we have shown that the inhibitory properties of EPO upon synaptic

501 transmission suffice to induce long-lasting changes in synaptic weights, which are

502 homeostatic, since they oppose the effects of aEPO. The increase in mEPSC frequency

503 and the decrease in mIPSC frequency detected when pEPO was followed by a washout

504 period (1h), are akin to the regulation of the excitatory-inhibitory balance previously

505 described for TTX-based protocols of activity deprivation (AD) (**Echegoyen et al.,**

506 **2007**). The use of TTX-based paradigms to induce homeostatic plasticity is well

507 established, as these have been extensively studied in recent years (reviewed, in detail,

508 by **Pozo and Goda, 2010**). Therefore, they can serve as a good experimental standard,

509 from which comparisons can be drawn, to further our understanding of the newly

510 identified EPO paradigm.

511 We have already identified two fundamental differences between these paradigms,

512 since, contrarily to what we have shown for EPO, prolonged exposure to TTX (48-60h)

513 was able to promote silent synapse formation in cultured hippocampal slices (**Arendt et**

514 **al., 2013**) and to increase sensitivity of cultured cortical PCs to injected current by

515 increasing voltage-dependent conductances (**Desai et al., 1999**).

516 Notwithstanding, further comparisons need to be drawn to attain a fuller picture,

517 particularly on the effects upon evoked transmission and hebbian plasticity phenomena.

518 Our main experimental model (acute hippocampal slices) does not allow for such

519 prolonged incubation periods with TTX. **Sutton et al. (2006)** showed that when TTX is

520   combined with blockade of postsynaptic NMDARs, synaptic scaling occurs an order of

521   magnitude faster (3h). We previously showed that this combined bath-applied

522   pharmacological AD protocol is able to upscale afferent-evoked excitatory transmission

523   and to lower the threshold for associative, input-specific potentiation of synaptic

524   strength (**Félix-Oliveira et al., 2014**).

525   Thus, we next aimed to evaluate the effect of EPO on evoked synaptic transmission and

526   to contrast it with that elicited by the AD protocol. To this end, we measured

527   extracellularly recorded input-output (I/O) curves (each variable was calculated as

528   detailed in **Figure S4A**). Consistent with previous results (**Félix-Oliveira et al., 2014**),

529   field responses were increased in AD slices (top parameter of fitted curves±s.e.:

530   veh:3.35 ± 0.20 mV/ms, AD:4.66 ± 0.36 mV/ms; n=8-9, $p=0.0241$, sum-of-square F-test;

531   **Figure 5A**). In stark contrast, pEPO decreased the field responses to afferent

532   stimulation (top parameter of fitted curves±s.e.: veh:3.35 ± 0.20 mV/ms, EPO:2.68 ± 0.14

533   mV/ms; n=9 per condition, $p=0.0161$, sum-of-square F-test; **Figure 5A**). Simultaneous

534   co-incubation with AD and pEPO (n=7) did not modify the I/O curve (*vs.* veh,

535     $p=0.3626$), thus suggesting that these modulatory effects are opposite, additive and

536   independent (**Figure 5A**). Importantly, these effects are unrelated to major changes in

537   the recruitment of presynaptic neurons, as the presynaptic fiber volley amplitude

538   (PFVA) was not significantly changed by EPO or AD (**Figure S4B**). When the fEPSP

539   slope is plotted as a function of the PFVA, similar results are rendered, although the

540   inhibitory effect of EPO (top parameter of fitted curves±s.e.: veh:3.75 ± 0.65 mV/ms,

541   EPO:2.91 ± 0.43 mV/ms; n=9 per condition, **Figure S4C**) did not reach statistical

542   significance ($p=0.3747$, sum-of-square F-test).

543   We also measured the amplitude of the population spike (popspike) whenever the

544   stimulus intensity was sufficient to elicit it (typically, above 220 μA stimulus intensity),

545 and plotted it against the corresponding fEPSP slope (**Figure 5B)**. The popspike

546 amplitude is a measure of CA1 pyramidal cell output, while the fEPSP slope evaluates

547 the response to Schaffer collateral fibers depolarization induced by an electrical

548 stimulus. Therefore, by correlating these two variables, we can assess how the

549 hippocampal circuit itself is computing afferent stimulation (**Rombo et al., 2015**).

550 Linear fittings of these values show no gross changes induced by either pEPO or AD

551 (**Figure 5B**). More detailed analysis of the results pertaining high stimulus intensities

552 shows a trend for change in popspike amplitude that closely reflects the changes in

553 fEPSP slope (**Figure S4D1**). Therefore, we also calculated the quotient between the

554 popspike amplitude and the fEPSP slope for all stimulus intensities and, accordingly, no

555 significant changes were observed (right inset of **Figure 5B**). Also, subgroup analysis

556 of the results according to stimulus intensity shows no differences between test

557 conditions (**Figure S4D2**). Altogether, these data suggest that the CA1 pyramidal cell

558 output to Schaffer collateral excitation is unaffected by either pEPO or AD.

559

560 *Activity deprivation and prolonged exposure to EPO prime hippocampal Long-Term*

561 *Potentiation (LTP) through additive mechanisms*

562 Next, we evaluated how prolonged exposure to EPO affects the capacity of CA1

563 neurons to undergo hebbian long-term potentiation (LTP). We elicited LTP at SC-CA1

564 synapses by delivering a weak θ-burst protocol (see **Materials and Methods**). LTP

565 magnitude following pEPO or AD treatment was significantly higher than in vehicle-

566 treated slices (veh:$19.99\pm2.70\%$; *vs* pEPO:$44.44\pm3.62\%$, *p*<0.01; *vs* AD:$47.83\pm3.56\%$,

567 *p*<0.01; n=9 per condition, one-way ANOVA with Sidak's multiple comparisons test;

568 **Figure 6B-C**). Furthermore, LTP elicited after co-incubation with pEPO and the AD

569 protocol was significantly higher than in slices incubated with pEPO or AD alone

570 (AD+pEPO: 69.01±10.36%, *p*<0.05 for both comparisons; n=7-9, one-way ANOVA

571 with Sidak's multiple comparisons test; **Figure 6C**). As previously shown (**Kamal et**

572 **al., 2011**), when EPO was applied only briefly before LTP induction (aEPO) it also

573 significantly increased LTP magnitude (veh:19.99±2.70%, n=9; *vs* aEPO:43.59±4.66%,

574 n=6, *p*<0.05, one-way ANOVA with Sidak's multiple comparisons test; **Figure 6D-E**).

575 However, aEPO did not increase LTP (or PTP) further after AD, yielding a significantly

576 lower LTP magnitude, as compared to the LTP attained after co-incubation with AD

577 and pEPO (AD+aEPO:45.33±4.31%, n=7; *vs* AD+pEPO:69.01±10.36%, n=7, *p*<0.05,

578 one-way ANOVA with Sidak's multiple comparisons test; **Figure 6D-E**). These results

579 indicate that the mechanisms underlying acute EPO-mediated boost of LTP are different

580 from the LTP priming-related changes that take place during the recovery period

581 following prolonged exposure to EPO.

582 Overall, these results also suggest that cumulative synaptic modifications are elicited by

583 prolonged exposure to EPO or AD, rendering them more prone to θ-burst-induced LTP.

584 One plausible explanation is that tuning of glutamatergic transmission by EPO would be

585 different in an activity-deprived network. However, in this regard, we found that the

586 effects of aEPO on mEPSCs and AMPA receptor trafficking were preserved after AD

587 (see **Figure S5A** and **Figure S5D**), although the downregulation of fEPSPs was lost

588 (see **Figure S5B**). Another possibility is that the synapses primed for LTP either in

589 activity deprived conditions or after prolonged exposure to EPO, recruit distinct and

590 independent mechanisms for LTP maintenance. Mature glutamatergic synapses are

591 thought to express only calcium-impermeable (CI), GluA2-containing AMPARs.

592 Contribution of calcium-permeable (CP), GluA2-lacking AMPAR to LTP is more

593 controversial. The current view is that CP-AMPARs are needed for θ-burst LTP

594 induction, but not for tetanus-induced LTP, nor for θ-burst LTP maintenance (**Plant et**

595   **al., 2006;** see also **Adesnik and Nicoll, 2007**). To evaluate if CP-AMPAR recruitment

596   could contribute to the higher LTP magnitudes attained after pEPO or after AD, we

597   delivered the same weak θ-burst protocol to two independent pathways and added the

598   specific CP-AMPAR extracellular blocker, NASPM (20 μM; **Blaschke et al., 1993**), to

599   the superfusion solution, 20 min after LTP induction in the second pathway. NASPM

600   significantly decreased the magnitude of LTP primed by pEPO, but not by AD

601   (pEPO+NASPM:30.90±2.49% *vs* AD+NASPM:52.56±4.80%, $p<0.01$;

602   pEPO:45.22±4.33% *vs* AD:49.71±3.10%, $p>0.05$; n=6-7, one-way ANOVA with

603   Bonferroni's correction; **Figure 7**). These data suggest that, unlike the mechanisms

604   engaged by activity deprivation, LTP primed by prolonged exposure to EPO requires

605   CP-AMPAR for LTP maintenance stage.

606

Discussion

Research over the last 20 years has highlighted roles for EPO far beyond erythropoiesis (**Fond et al., 2012**). Although data from several cohorts uphold the inkling that EPO acts as a cognitive enhancer, its actions on synaptic function and plasticity have remained elusive.

The work herein described is the first to show that EPO is able to elicit homeostatic plasticity phenomena (**Supplementary Table 1**). In this regard, our results revealed that acute EPO acts as an inhibitor of excitatory synaptic transmission, while prolonged exposure to EPO (if followed by a recovery period) produces opposite changes, i.e. an upregulation of spontaneous miniature excitatory events. Homeostatic scaling was first described as a slow compensatory upscaling of synaptic strength secondary to loss of AP-driven neurotransmission by long-term (24h) exposure to TTX (**Turrigiano et al., 1998**). Subsequent work showed that when TTX was combined with blockade of NMDAR-mediated responses to stochastic quantal glutamate release, homeostatic adjustment of spontaneous transmission occurred an order of magnitude faster (3h) (**Sutton et al., 2006**). Here, for the first time, we describe a paradigm that induces similar homeostatic changes in spontaneous transmission, while leaving AP-driven events and the number of silent synapses intact. These homeostatic changes require, nonetheless, a recovery period (1h), in EPO-free aCSF. Such dependence on a reactivation stage has been previously noted for the homeostatic changes in excitatory transmission induced by TTX (**Gerkin et al., 2013**).

While evaluating spontaneous (AP-dependent and independent) synaptic activity in mice chronically treated with EPO, **Adamcio et al., (2008)** reported an increase in inhibitory and a decrease in excitatory transmission, which is in apparent conflict with our observations on mEPSC, mIPSC and sEPSC scaling. This discrepancy may be due

to species- and/or age-related differences in EpoR expression, which progressively decreases postnatally (**Sanchez et al., 2009**). Furthermore, chronic EPO administration *in vivo* (**Adamcio et al., 2008**) may lead to receptor desensitization and/or affect receptor coupling to downstream transduction pathways.

When studying afferent-evoked synaptic transmission, we observed that, unlike AD (**Félix-Oliveira et al., 2014**), not only does prolonged EPO fail to potentiate, as it decreases the input-output relationship. This inhibition could reflect the reduction in the readily releasable pool size shown to occur after prolonged exposure of autaptic neuronal cultures to a low concentration of EPO (**Adamcio et al., 2008**). Alternatively, it can be directly linked to the upscaling in spontaneous transmission. Indeed, recent studies challenge the classical association between the fusion rates of spontaneous- and activity-dedicated vesicles, by showing that these two forms of release may be independent (**Melom et al., 2013**) or may even correlate inversely (**Peled et al., 2014**).

Intriguingly, EPO attains lasting and opposite modifications of both evoked and spontaneous transmission, with only the latter corresponding to a compensatory homeostatic upscaling. In contrast, AD homeostatically upregulates both spontaneous and electrically-evoked transmission (**Félix-Oliveira et al., 2014**). This discrepancy likely relates to a fundamental difference between both paradigms. EPO mainly silences stochastic glutamatergic inputs onto their postsynaptic contacts, while not affecting AP generation. Hence, it affords a more selective modulation of the spontaneous release (not AP-driven) vesicle pool. In contrast, the AD paradigm (**Sutton et al., 2006**) attains a much stronger but less selective inhibition of excitatory transmission. Therefore, while our data support that AP blockade is not an absolute requirement for the rapid scaling of miniature transmission, we also demonstrate that the consequences for synaptic function are not independent of AP blockade. Another advantage in having contrasted EPO with

657   the AD paradigm is that it allowed us to address how EPO affects synaptic transmission

658   and plasticity of activity deprived neurons. Such data is timely, given that EPO is

659   currently considered a potential therapeutic option for patients with ischemic stroke

660   (e.g., **Cramer et al., 2014**; **Ehrenreich et al, 2009**). Indeed, the present experimental

661   conditions may resemble those of neurons that remain viable after a stroke event, albeit

662   suffering from activity deprivation due to loss of input from ischemic afferent neurons.

663   Overall, our data also support the emerging view that spontaneous and activity-

664   dependent pools of vesicles are spatially and functionally segregated and may, thus, be

665   independently regulated (**Kavalali, 2015**). Our results further suggest that EPO acts

666   mainly through presynaptic targets, putatively through one or more of the proteins that

667   comprise the vesicle release machinery preferentially devoted to spontaneous release

668   (e.g., **Chung et al., 2010**). In this regard, presynaptic-derived forms of homeostatic

669   plasticity have been previously described (e.g., **Murthy et al., 2001**).

670   Despite their differences, both prolonged EPO and AD prime plasticity at the

671   hippocampus. LTP has been reported to increase after acute superfusion with EPO

672   (**Kamal et al., 2011**) and following chronic EPO injections (**Adamcio et al., 2008**).

673   However, the underlying molecular mechanisms in both time frames seem to be

674   different, as we herein show that, unlike prolonged EPO, acute EPO-driven

675   enhancement of LTP is not cumulative with previous AD. On the one hand, LTP

676   requires intracellular calcium to rise in specific subcellular compartments beyond a

677   critical threshold (**Citri and Malenka, 2008**) and EPO can acutely activate calcium

678   channels, rapidly increasing intracellular Ca^{2+} (**Koshimura et al., 1999**). On the other

679   hand, we now show that prolonged EPO causes a homeostatic response in spontaneous

680   transmitter release, which, in and of itself, suffices to elicit postsynaptic plasticity

681 (**Nosyreva et al., 2013**) and has also been shown to mediate the rapid antidepressant

682 action of ketamine (**Autry et al., 2011; Nosyreva et al., 2013**).

683 In keeping with the idea that prolonged EPO and AD do not engage the same

684 mechanisms to boost plasticity is the finding that, when co-applied, EPO and AD have

685 an additive influence over LTP. Moreover, pEPO- and AD-primed LTP are maintained

686 through different processes. Vehicle-treated and AD-primed LTPs are not affected by

687 superfusion with a specific CP-AMPAR inhibitor during their consolidation stage,

688 supporting prior evidence that these receptors do not contribute for LTP maintenance

689 (**Plant et al., 2006**). In contrast, prior treatment with pEPO leads to an enhanced LTP

690 that is sensitive to CP-AMPAR blockade. Although GluA2-lacking CP-AMPA

691 receptors are virtually excluded from adult synapses (**Henley and Wilkinson, 2016**),

692 integration of relevant stimuli can recruit them from extrasynaptic pools into the

693 synaptic compartment (**Oh et al., 2006**), where a proportionally small increase in CP-

694 AMPARs (~5%) is enough to afford the facilitation of synaptic efficiency that is

695 obtained in θ-burst LTP (**Guire et al., 2008**). Our results thus provide the first evidence

696 that mature synapses, under specific circumstances, may recruit CP-AMPARs beyond

697 the induction stage and into LTP maintenance. Future work will elucidate if other

698 paradigms with similar consequences for presynaptic release can engage CP-AMPAR-

699 dependent mechanisms for LTP consolidation or, alternatively, if this is an effect

700 specifically elicited by EPO. However, we cannot exclude that other, concurrent

701 mechanisms also underlie the cumulative effect of prolonged EPO and AD on LTP

702 magnitude. Indeed, long term (60h) incubation with TTX was shown to enhance LTP

703 by inducing silent synapse formation (**Arendt et al., 2013**), contrarily to what our

704 results suggest for EPO (**Figure 4D-G**).

705   In conclusion, our data show that EPO acts predominantly as an excitatory transmission

706   inhibitor, acutely downtuning spontaneous and, to a lesser extent, afferent-evoked

707   neurotransmission. Consistently, prolonged exposure to EPO, when followed by a

708   recovery period, selectively elicits homeostatic changes in spontaneous but not in

709   evoked transmission, while leaving neuronal intrinsic properties and the proportion of

710   silent synapses intact. EPO also primes synapses to undergo associative plasticity,

711   recruiting a non-canonical CP-AMPAR-dependent mechanism for LTP maintenance.

712   Collectively, our results represent a significant advance toward the understanding of the

713   synaptic basis for EPO's pro-cognitive effects and challenge some currently prevailing

714   views on fundamental principles of synaptic transmission and plasticity.

715

Authors' Contributions

R.B.D., T.M.R. and A.M.S. conceived the experiments. R.B.D. performed the patch-clamp recordings and the live imaging experiments, with contributions from D.M.R., J.R. and J.McG. T.M.R. performed the extracellular recordings. C.R.O. and A.F.R. prepared the primary hippocampal cultures. A.F.R. performed the immunocytochemistry procedures. R.B.D., T.M.R., D.M.R. and A.F.R. analyzed the experiments. R.B.D. and T.M.R. wrote the first draft of the manuscript, which was subsequently revised by A.M.S. and J.M.H.

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954 **Tables**

955

Table 1. Measures of intrinsic excitability of hippocampal CA1 pyramidal cells.			
	Vehicle (n = 15)	pEPO (n=15)	$p*$
Resting Membrane Potential (V_m) (mV)	−59.34 ± 1.22	−58.30 ± 1.25	0.5576
AP firing threshold ($\Delta V_m − V_t$)	7.53 ± 1.51	6.18 ± 1.82	0.5356
Delay to first AP (ms)	84.19 ± 7.89	74.92 ± 6.70	0.3812
Δt (AP1 to AP2) (ms)	88.46 ± 17.42	102.70 ± 25.35	0.6442
Maximum AP firing frequency (Hz)	33.87 ± 1.13	36.27 ± 1.76	0.2599

956 * Unpaired t-test

957

Figure Captions

Figure 1. Acute EPO (aEPO) presynaptically downregulates afferent-evoked transmission at SC-CA1 synapses, with no concurrent changes in postsynaptic excitability.

(A) Schematic representation of orthodromic fEPSP recordings at SC-CA1 synapses.

(B) Panel (B1) represents the time course of averaged normalized changes in fEPSP slope induced by superfusion with vehicle or EPO (aEPO, 2.4 IU/ml) (n=10 per condition). Panel (B2) shows the time course of average normalized changes in fEPSP slope in slices superfused with aEPO (2.4 IU/ml) in the presence of a tyrosine kinase inhibitor, k252a (200 mM), which was applied 30 min before EPO (n=5); control data from slices exposed only to k252a (200 mM) for the same amount of time is also shown (n=5). Data represented as mean ± s.e.m. *Top insets:* tracings obtained from representative experiments, as indicated, before (black) and 60 min after (gray) vehicle/aEPO superfusion.

(C) Histogram shows percentage changes in the average slope of the fEPSP, for the recordings in (B). Data represented as mean ± s.e.m. ****$p<0.0001$, as compared to vehicle; [pp] $p<0.01$, [ppp] $p<0.001$, as compared to aEPO perfusion alone (one-way ANOVA with Tukey's multiple comparisons test).

(D) PPF values for each individual experiment, with a line connecting averaged PPF values at baseline and after 40 min, in slices perfused with vehicle (Veh) (n=5, left panel) or with aEPO (2.4 IU/ml) (n=5, right panel). Histograms depict mean ± s.e.m. for each set of values. ** $p<0.01$, *n.s.* not significant (paired t-test) *Right inset:* tracings from representative experiments, as indicated, before (black) and 40 min after (gray) vehicle/aEPO application.

983 **(E)** Schematic representation of antidromic population spike (popspike) recordings at

984 the pyramidal cell layer.

985 **(F)** Time course of averaged normalized changes in popspike amplitude induced by

986 superfusion with vehicle (n=5) or aEPO (2.4 IU/ml, n=6). Data represented as mean ±

987 s.e.m. *Top insets:* tracings obtained from representative experiments, as indicated,

988 before (black) and 60 min after (gray) vehicle/aEPO superfusion.

989 **(G)** Schematic representation of intracellular recordings in current-clamp (CC) mode

990 from CA1 pyramidal cells (PC).

991 **(H)** Box and whiskers depicting the distributions of averaged AP firing frequencies

992 recorded in current-clamp mode, during superfusion with either vehicle (Veh) (n=9) or

993 aEPO (2.4 IU/ml) (n=6). *n.s.* not significant (Mann-Whitney test). *Right inset:*

994 representative tracings.

995

996 **Figure 2. Acute EPO (aEPO) superfusion does not affect AMPAR trafficking at**

997 **dendritic spines.**

998 **(A)** The left panels in (A1) and (A2) depict the time courses of normalized changes in

999 SEP-GluA2 fluorescence of punctate and diffuse surface GluA2 signal, respectively

1000 (n=3-4 ROI per neuron; 6 neurons DIC 17-24, from 2 independent cultures). The right

1001 panels in (A1) and (A2) depict the averaged fluorescence calculated at baseline and in

1002 the last 9 min of aEPO perfusion, with a line connecting averaged values from the same

1003 experiment (n=6). In this set of experiments, EPO was applied for only 12 min to

1004 minimize GluA2 fluorescence bleaching. Data represented as mean ± s.e.m. *n.s.* not

1005 significant (paired t-test). The representative dendrite in (A3) illustrates where the

1006 punctate (Spine, Sp) and diffuse (Dif) signals were measured.

1007   **(B-C)** Representative images of cultured hippocampal neurons at either DIC 15 (B) or

1008   DIC 21 (C) stained for the dendritic marker MAP2 (red) (B1, C1) and the EpoR protein

1009   (green) (B2, C2). The panels below (B) and (C) depict virtual images of the neurons in

1010   the main panels, generated by the ImageJ software, to exemplify the quantification of

1011   the mean gray values. Through the identification of the neuronal area on the MAP2

1012   channel (white painted area on the left virtual image from the MAP2 channel) and cell

1013   body region (red polygonal line on the right virtual image from the MAP2 channel), the

1014   two ROIs (cell body area and processes area) were generated to quantify the EpoR

1015   signal intensity in the cell body (white painted area on the left virtual image from the

1016   EpoR channel) and in dendrites (white painted area on the right virtual image from the

1017   EpoR channel). Scale bar = 30μm.

1018   **(D)** Histogram depicting the average mean gray values of EpoR protein signal intensity

1019   in the cell body (Soma) and in the dendrites (Dend), either at DIC 15 or DIC 21, as

1020   indicated below each bar. Data are expressed as mean ± s.e.m. from 60 neurons, taken

1021   from 2 independent cultures (30 neurons analyzed per culture) ***$p<0.0001$ (paired t-

1022   test).

1023

1024   **Figure 3. Prolonged EPO (pEPO) drives homeostatic changes in miniature**

1025   **excitatory and inhibitory synaptic events, opposite to its acute effects (aEPO), only**

1026   **if exposure is followed by a recovery period.**

1027   **(A-B)** Schematic representation of mEPSC (A) and mIPSC recordings from CA1

1028   pyramidal cells (PC). IN. Interneurons. VC. Voltage-clamp.

1029   **(D-E)** Panels in (D) and (E) depict the values of frequency (left panels), amplitude

1030   (central panels) and decay (right panels) of mEPSC (D) and mIPSC (E) events, which

1031   are plotted with a line connecting values at baseline and after 40 min superfusion with

aEPO (2.4 IU/ml) (n=7 for mEPSC recordings; n=5 for mIPSC recordings). Histograms

represent mean ± s.e.m. for each dataset. * *p*<0.05, ** *p*<0.01, *n.s.* not significant

(paired t-test). *Top insets:* representative mEPSC and mIPSC tracings, at baseline and

after aEPO (2.4 IU/mL) superfusion, are shown in panels (D) and (E), respectively.

(G-H) Histograms depict the frequency (left panels), amplitude (central panels) and

decay (right panels) of mEPSC (G, n=5-8 per condition) and mIPSC (H, n=9 per

condition) events recorded from CA1 PCs of hippocampal slices incubated for 3h with

either vehicle (black) or pEPO (2.4 IU/ml, gray) and thereafter continuously superfused

with EPO (2.4 IU/ml), in order to avoid recovery. Data represented as mean ± s.e.m. *

p<0.05, *n.s.* not significant (unpaired t-test). *Top insets*: representative mEPSC (G) and

mIPSC (H) tracings, for each condition.

(J-K) Histograms depict the frequency (left panels), amplitude (central panels) and

decay (right panels) of mEPSC (J, n=11 per condition) and mIPSC (L, n=6-7 per

condition) events recorded from CA1 PCs of hippocampal slices incubated for 3h with

either vehicle (black) or pEPO (2.4 IU/ml, gray), followed by a 1h recovery period.

Data represented as mean ± s.e.m. * *p*<0.05, *n.s.* not significant (unpaired t-test with

Welch's correction for mEPSC data; unpaired t-test mIPSC data). *Top insets*:

representative mEPSC (J) and mIPSC (K) tracings, for each condition.

Schematic representations of the experimental designs for acute EPO application **(C)**

and prolonged EPO exposure [either with **(I)** or without **(F)** a recovery/washout (Wsh)

period] are shown.

Figure 4. Prolonged EPO (pEPO) does not affect postsynaptic membrane

excitability, nor the proportion of silent synapses.

(**A**) Schematic representation of intracellular recordings in current-clamp (CC) mode

from CA1 pyramidal cells (PC).

(**B**) Instantaneous AP firing frequency is plotted as a function of injected current

for CA1 PCs from hippocampal slices treated with pEPO (2.4 IU/ml) or vehicle for 3h

(n=15 per condition), followed by a 1h recovery period. Data represented as mean ±

s.e.m. with non-linear fittings.

(**C**) Representative AP firing pattern (left) and maximum AP firing (right) tracings are

shown for each condition (vehicle-treated in black; pEPO-treated in gray).

(**D**) Schematic representation of intracellular recordings from CA1 pyramidal cells (PC)

of electrically evoked AMPA- and NMDA-mediated responses.

(**E**) Panel depicts the quantification of failure rate of evoked responses for vehicle-

(n=9) and pEPO-treated (n=8) cells. Individual experiments are plotted with a line

connecting failure rate values at −60 and +40 mV holding potentials. Histograms

represent mean ± s.e.m. for each dataset. Failure rate is calculated from 50 sweeps per

cell. *n.s.* not significant (paired t-test)

(**F-G**) Example tracings and scatter plot of evoked EPSCs from one vehicle- (F) and

one pEPO-treated (G) neuron at −60 and +40 mV holding potentials. Failed events were

assigned an amplitude of zero for ease of visualization.

Figure 5. Prolonged EPO (pEPO) downregulates afferent-evoked synaptic

transmission.

(**A**) Input/Output curves from vehicle-, AD-, pEPO- and both AD/pEPO-treated slices

(n=7-9 per condition), where the fEPSP slope is plotted as a function of stimulus

1081 intensity. Data represented as mean ± s.e.m. with non-linear fittings. *Right inset:*

1082 histogram depicting the top parameter of the fitted curves in (A) for each condition ±

1083 standard error. *$p<0.05$ (sum-of-squares F-test). *Bottom inset:* representative tracings

1084 are shown for each condition depicting evoked responses to 180, 240 and 300 µA

1085 stimulations.

1086 **(B)** Population spike amplitude is plotted as a function of the fEPSP slope; data from all

1087 the field responses in which the population spike was elicited were included. The lines

1088 represent linear fittings (n=7-9 per condition). *Right inset:* histogram depicting the

1089 popspike amplitude / fEPSP slope ratio. Data represented as mean ± s.e.m. n.s. not

1090 significant (one-way ANOVA with Bonferroni's correction).

1091

1092 **Figure 6. Prolonged EPO (pEPO) and AD cumulatively boost hippocampal Long-**

1093 **Term Potentiation (LTP).**

1094 **(A)** Schematic representation of fEPSP recordings at SC-CA1 synapses (A1). The weak

1095 θ-burst LTP-inducing protocol (A2) was delivered by S1.

1096 **(B-D)** Experimental designs are respectively shown in (B1), (C1) and (D1). Panels (B2)

1097 and (C2) depict the time courses of averaged normalized changes in fEPSP slope after

1098 delivery (arrow) of a weak θ-burst (1x4x4) to vehicle-treated or AD-treated

1099 hippocampal slices (B2, n=9 per condition), and to pEPO-treated or AD+pEPO-treated

1100 slices (C2, n=7-9 per condition). Panel (D2) depicts the time course of averaged

1101 normalized changes in fEPSP slope after delivery (arrow) of a weak θ-burst (1x4x4) to

1102 vehicle-treated and AD-treated slices (n=6-7 per condition), but where aEPO (2.4

1103 IU/ml) was only present in the superfusion solution, at least 60 min before LTP

1104 induction and was kept for the 60 min subsequent to LTP induction. Data represented as

1105   mean ± s.e.m. *Right insets*: representative tracings, as indicated, before (thin line) and

1106   60 min after (thicker line) θ-burst delivery.

1107   **(E)** Histograms depict averaged LTP **(E1)** and PTP magnitudes **(E2)**, for the recordings

1108   shown in **(B-D)**. Data represented as mean ± s.e.m. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$,

1109   as compared to vehicle; $^{\rho}$ $p < 0.05$, as compared to AD+pEPO (one-way ANOVA with

1110   Sidak's multiple comparisons test).

1111

1112   **Figure 7. Prolonged EPO (pEPO) recruits calcium-permeable AMPA receptors**

1113   **(CP-AMPAR) for LTP maintenance.**

1114   **(A)** Schematic representation of fEPSP recordings at SC-CA1 synapses (A1). The weak

1115   θ-burst LTP-inducing protocol was delivered to two independent pathways (S1 and S2).

1116   The experimental design is shown in (A2).

1117   **(B)** The top panels represent the time course of changes in fEPSP slope after delivery

1118   (arrow) of a weak θ-burst (1x4x4) to both pathways of vehicle- **(B1)**, pEPO- **(B2)** and

1119   AD-treated **(B3)** slices (n=6-7 per condition). NASPM (20 μM) superfusion began 20

1120   min after LTP induction in the 2nd pathway (full circles), which was induced at least 60

1121   min following LTP induction in the 1st pathway (open circles). The bottom panels show

1122   the time course of averaged normalized changes in fEPSP slope in the 2nd pathway

1123   during LTP induction in the 1st pathway. Data represented as mean ± s.e.m.

1124   **(C)** Histogram depicting averaged LTP magnitudes, for the recordings in (B) (n=6-7 per

1125   condition). Data represented as mean ± s.e.m. **$p < 0.01$, ***$p < 0.001$, as compared to

1126   vehicle; $^{\varphi\varphi}$ $p < 0.01$, $^{\varphi\varphi\varphi}$ $p < 0.001$, as compared to AD (one-way ANOVA with

1127   Bonferroni's correction).

1128

1129