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2 Erythropoietin induces homeostatic plasticity at
3 hippocampal synapses
4

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28 **Running title:** Erythropoietin tuning of hippocampal synapses
29

30 **Abstract**

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

48

49

50

51 **Keywords:** Erythropoietin; hippocampus; homeostatic plasticity; long-term
52 potentiation; spontaneous and evoked neurotransmission.

53 **Introduction**

54 Erythropoietin (EPO) is a pleiotropic hormone from the type I cytokine super-family
55 best known as a crucial regulator of erythropoiesis and tissue oxygenation homeostasis.
56 In addition, EPO signaling has been shown to play key roles in the central nervous
57 system (CNS), where neurons and astrocytes express a specific non-hematopoietic EPO
58 receptor (**Fond et al., 2012**). EPO is an important regulator of neuronal proliferation
59 and migration during CNS development and also following injury to the mature brain,
60 such as during post-stroke recovery (**Tsai et al., 2006**). Intriguingly, in addition to its
61 role in neurogenesis, early studies reported positive cognitive effects in patients
62 regularly receiving EPO for chronic kidney disease (**Kambova, 1998; Pickett et al.,**
63 **1999**). Similar pro-cognitive effects have since been reported in patients with
64 schizophrenia (**Ehrenreich et al., 2007**), bipolar disorder (**Miskowiak et al., 2014**) and
65 type 1 diabetes (**Kristensen et al., 2013**).

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

73 In keeping with these data, recent evidence indicates that EPO directly modulates
74 neuronal activity and plasticity (**Adamcio et al., 2008; Kamal et al., 2011; Sargin et**
75 **al., 2011**). However, differences in the experimental models used to investigate EPO
76 signaling have yielded some inconsistencies in results (**Supplementary Table 1**). We
77 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 μ M), a sodium channel blocker, and APV (50 μ M), 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

94 **Materials and Methods**

95

96 Animals

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

103

104 Primary Hippocampal Cultures

105 Pregnant Sprague-Dawley rats were obtained from Harlan Interfauna Iberia (Barcelona,
106 Spain). Animals were deeply anesthetized with halothane (2-Bromo-2-Chloro-
107 1,1,1-Trifluoroethane) in an anesthesia chamber before being sacrificed by decapitation.
108 Briefly, embryos (E17-E18) were rapidly removed from the uterus, their brains removed
109 and placed in cold Ca²⁺- and Mg²⁺-free Hank's Balanced Salt Solution supplemented
110 with 0.37% glucose (HBSS-glucose). After brain isolation, meninges were gently
111 removed from the hemispheres. All hippocampi were collected in fresh HBSS-glucose
112 and trypsinization was carried out (0,350 ml of 2,5% trypsin) at 37 °C for 15 min in a
113 water bath. Afterwards, trypsin solution was gently removed and 30% Fetal Bovine
114 Serum (FBS) in HBSS-glucose was added and let stand for 5 min at room temperature
115 (RT, 22-24°C) to quench trypsin activity. The pellet was resuspended in Neurobasal
116 medium (Neurobasal-B27: Neurobasal supplemented with 0.5 mM glutamine, 2% B27,
117 25 U/mL penicillin/streptomycin) and 25 mM glutamic acid. Cell suspension was
118 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/cm² 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 37°C and 5% CO₂
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 CaCl₂; 7 MgCl₂; 25 NaHCO₃; 1.25 NaH₂PO₄; and 7 glucose; previously
130 gassed with 95% O₂ and 5% CO₂, 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 NaH₂PO₄; 26 NaHCO₃; 1 MgSO₄; 2 CaCl₂; and 10 glucose), previously
136 gassed with 95% O₂ and 5% CO₂, 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 32°C. Evoked orthodromic field excitatory
154 postsynaptic potentials (fEPSP) were recorded extracellularly through a microelectrode
155 filled with 4 M NaCl (2–8 M Ω 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 μ A 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 μ A to a supramaximal stimulation of 320 μ A. 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 μ M) 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 M Ω
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

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

230

231 *Patch-clamp Recordings.*

232 Whole cell patch-clamp recordings were obtained either from hippocampal CA1
233 pyramidal cells or from hippocampal cultured neurons, as indicated. These were
234 visualized with an upright microscope (Zeiss Axioskop 2FS) equipped with infrared
235 video microscopy and differential interference contrast optics. Recordings were
236 performed at RT in current-clamp or voltage-clamp mode with an Axopatch 200B
237 (Axon Instruments) amplifier. For current-clamp recordings and for miniature or
238 spontaneous excitatory postsynaptic current (mEPSC or sEPSC, respectively)
239 recordings, patch pipettes (4- to 7-M Ω resistance) were filled with an internal solution
240 containing (in mM): 125 K-gluconate, 11 KCl, 0.1 CaCl₂, 2 MgCl₂, 1 EGTA, 10
241 HEPES, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine, pH 7.3, adjusted with 1 M
242 KOH, 280–290 mOsm. For miniature inhibitory postsynaptic current (mIPSC)
243 recordings and AMPA- and NMDA-mediated responses, the internal solution contained

244 (in mM): 125 CsCl, 8 NaCl, 1 CaCl₂, 10 EGTA, 10 HEPES, 10 glucose, 5 MgATP, and
245 0.4 NaGTP, pH 7.2, adjusted with CsOH (50 wt% in H₂O), 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 (V_h) 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 μ M) 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 μ M).
275 Synaptic responses were obtained with a bipolar electrode placed over Schaffer
276 collateral fibers and stimuli (100 μ s) 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 35°C) 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). NH₄Cl (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 KH₂PO₄, 10 Na₂HPO₄·2H₂O, 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 ($A \cap B$), the dendrites ($A \setminus B$) and the background ($C \setminus A$). 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.

342 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 \pm 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.

372 **Results**

373

374 *EPO acutely (aEPO) downregulates evoked transmission by operating presynaptic*

375 *mechanisms*

376 We first reexamined the effect of acute EPO (aEPO) superfusion on afferent-evoked
377 transmission to CA1 neurons, by eliciting orthodromic fEPSPs in hippocampal slices.

378 We found that aEPO significantly inhibited fEPSP slope (**Figure 1B1**) even when
379 applied at a lower, more physiological concentration (2.4 IU/ml) than previously used
380 (50 IU/ml in **Kamal et al., 2011**). EPO signals through EPO receptors (EpoRs), which
381 are frequently found as homodimeric tyrosine kinase receptors (RTKs) in the CNS
382 (**Digicaylioglu and Lipton, 2001; Ma et al., 2016**). As shown in **Figure 1B2-C**, the
383 effect of EPO was prevented by the tyrosine kinase inhibitor, k252a (200 nM), thus
384 suggesting that EPO downregulates afferent-evoked transmission at SC-CA1 synapses
385 through homodimeric EpoRs. Interestingly, we found a consistent time lag of
386 approximately 10 minutes before aEPO's effect became apparent (**Figure 1B1**). This
387 lag was described before by **Kamal et al., 2011** and may relate to the fact that none of
388 the known signaling molecules downstream to the EpoR are able to directly modulate
389 membrane ionic currents (**Ma et al., 2016**); thus, additional steps (e.g., ion channel
390 membrane trafficking or degradation) may be required.

391 In order to further dissect the effect of acutely applied EPO on activity-dependent
392 synaptic transmission, namely to determine whether it should be ascribed to pre- and/or
393 postsynaptic mechanisms, we performed a paired-pulse facilitation (PPF) protocol,
394 evoked antidromic responses from CA1 neurons and recorded spontaneous changes in
395 membrane potential intracellularly from CA1 pyramidal cells (PCs) soma. We found
396 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 (AMPA) 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 \pm s.e.:
477 veh:34.89 \pm 1.50 Hz, EPO:37.68 \pm 2.27 Hz, $p>0.05$; slopes of fitted curves \pm s.e.:
478 veh:0.0096 \pm 0.0018 Hz/pA, EPO:0.0094 \pm 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 \pm 3.65%; +40 mV:37.33 \pm 7.48%; n=9,
492 $p=0.4192$, paired t-test) and in pEPO-treated slices (-60 mV:43.25 \pm 6.58%; +40
493 mV:46.75 \pm 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 \pm s.e.:
530 veh:3.35 \pm 0.20 mV/ms, AD:4.66 \pm 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 \pm s.e.: veh:3.35 \pm 0.20 mV/ms, EPO:2.68 \pm 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 \pm s.e.: veh:3.75 \pm 0.65 mV/ms,
541 EPO:2.91 \pm 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 μ A 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: $19.99 \pm 2.70\%$; vs pEPO: $44.44 \pm 3.62\%$, $p < 0.01$; vs AD: $47.83 \pm 3.56\%$,
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: $69.01 \pm 10.36\%$, $p < 0.05$ 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: $19.99 \pm 2.70\%$, $n = 9$; vs aEPO: $43.59 \pm 4.66\%$,
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: $45.33 \pm 4.31\%$, $n = 7$; vs AD+pEPO: $69.01 \pm 10.36\%$, $n = 7$, $p < 0.05$,
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-AMPA receptors 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-AMPA 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-AMPA extracellular blocker, NASPM (20 μ M; **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 \pm 2.49% vs AD+NASPM:52.56 \pm 4.80%, p <0.01;
602 pEPO:45.22 \pm 4.33% vs AD:49.71 \pm 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-AMPA for LTP maintenance stage.
606

607 **Discussion**

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

612 The work herein described is the first to show that EPO is able to elicit homeostatic
613 plasticity phenomena (**Supplementary Table 1**). In this regard, our results revealed that
614 acute EPO acts as an inhibitor of excitatory synaptic transmission, while prolonged
615 exposure to EPO (if followed by a recovery period) produces opposite changes, i.e. an
616 upregulation of spontaneous miniature excitatory events. Homeostatic scaling was first
617 described as a slow compensatory upscaling of synaptic strength secondary to loss of
618 AP-driven neurotransmission by long-term (24h) exposure to TTX (**Turrigiano et al.,**
619 **1998**). Subsequent work showed that when TTX was combined with blockade of
620 NMDAR-mediated responses to stochastic quantal glutamate release, homeostatic
621 adjustment of spontaneous transmission occurred an order of magnitude faster (3h)
622 (**Sutton et al., 2006**). Here, for the first time, we describe a paradigm that induces
623 similar homeostatic changes in spontaneous transmission, while leaving AP-driven
624 events and the number of silent synapses intact. These homeostatic changes require,
625 nonetheless, a recovery period (1h), in EPO-free aCSF. Such dependence on a
626 reactivation stage has been previously noted for the homeostatic changes in excitatory
627 transmission induced by TTX (**Gerkin et al., 2013**).

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

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

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

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

716 **Authors' Contributions**

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

724

725

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737

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954 **Tables**

955

Table 1. Measures of intrinsic excitability of hippocampal CA1 pyramidal cells.			
	Vehicle (n = 15)	pEPO (n=15)	<i>p</i> *
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

958 **Figure Captions**

959

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

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

964 (B) Panel (B1) represents the time course of averaged normalized changes in fEPSP

965 slope induced by superfusion with vehicle or EPO (aEPO, 2.4 IU/ml) (n=10 per

966 condition). Panel (B2) shows the time course of average normalized changes in fEPSP

967 slope in slices superfused with aEPO (2.4 IU/ml) in the presence of a tyrosine kinase

968 inhibitor, k252a (200 mM), which was applied 30 min before EPO (n=5); control data

969 from slices exposed only to k252a (200 mM) for the same amount of time is also shown

970 (n=5). Data represented as mean \pm s.e.m. *Top insets:* tracings obtained from

971 representative experiments, as indicated, before (black) and 60 min after (gray)

972 vehicle/aEPO superfusion.

973 (C) Histogram shows percentage changes in the average slope of the fEPSP, for the

974 recordings in (B). Data represented as mean \pm s.e.m. **** $p < 0.0001$, as compared to

975 vehicle; ^{pp} $p < 0.01$, ^{ppp} $p < 0.001$, as compared to aEPO perfusion alone (one-way

976 ANOVA with Tukey's multiple comparisons test).

977 (D) PPF values for each individual experiment, with a line connecting averaged PPF

978 values at baseline and after 40 min, in slices perfused with vehicle (Veh) (n=5, left

979 panel) or with aEPO (2.4 IU/ml) (n=5, right panel). Histograms depict mean \pm s.e.m. for

980 each set of values. ** $p < 0.01$, *n.s.* not significant (paired t-test) *Right inset:* tracings

981 from representative experiments, as indicated, before (black) and 40 min after (gray)

982 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 \pm
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 \pm 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 = 30 μ m.

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 \pm 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

1032 aEPO (2.4 IU/ml) (n=7 for mEPSC recordings; n=5 for mIPSC recordings). Histograms
1033 represent mean \pm s.e.m. for each dataset. * $p<0.05$, ** $p<0.01$, *n.s.* not significant
1034 (paired t-test). *Top insets*: representative mEPSC and mIPSC tracings, at baseline and
1035 after aEPO (2.4 IU/mL) superfusion, are shown in panels (D) and (E), respectively.
1036 **(G-H)** Histograms depict the frequency (left panels), amplitude (central panels) and
1037 decay (right panels) of mEPSC (G, n=5-8 per condition) and mIPSC (H, n=9 per
1038 condition) events recorded from CA1 PCs of hippocampal slices incubated for 3h with
1039 either vehicle (black) or pEPO (2.4 IU/ml, gray) and thereafter continuously superfused
1040 with EPO (2.4 IU/ml), in order to avoid recovery. Data represented as mean \pm s.e.m. *
1041 $p<0.05$, *n.s.* not significant (unpaired t-test). *Top insets*: representative mEPSC (G) and
1042 mIPSC (H) tracings, for each condition.
1043 **(J-K)** Histograms depict the frequency (left panels), amplitude (central panels) and
1044 decay (right panels) of mEPSC (J, n=11 per condition) and mIPSC (L, n=6-7 per
1045 condition) events recorded from CA1 PCs of hippocampal slices incubated for 3h with
1046 either vehicle (black) or pEPO (2.4 IU/ml, gray), followed by a 1h recovery period.
1047 Data represented as mean \pm s.e.m. * $p<0.05$, *n.s.* not significant (unpaired t-test with
1048 Welch's correction for mEPSC data; unpaired t-test mIPSC data). *Top insets*:
1049 representative mEPSC (J) and mIPSC (K) tracings, for each condition.
1050 Schematic representations of the experimental designs for acute EPO application (C)
1051 and prolonged EPO exposure [either with (I) or without (F) a recovery/washout (Wsh)
1052 period] are shown.
1053
1054
1055

1056 **Figure 4. Prolonged EPO (pEPO) does not affect postsynaptic membrane**
1057 **excitability, nor the proportion of silent synapses.**

1058 (A) Schematic representation of intracellular recordings in current-clamp (CC) mode
1059 from CA1 pyramidal cells (PC).

1060 (B) Instantaneous AP firing frequency is plotted as a function of injected current
1061 for CA1 PCs from hippocampal slices treated with pEPO (2.4 IU/ml) or vehicle for 3h
1062 (n=15 per condition), followed by a 1h recovery period. Data represented as mean \pm
1063 s.e.m. with non-linear fittings.

1064 (C) Representative AP firing pattern (left) and maximum AP firing (right) tracings are
1065 shown for each condition (vehicle-treated in black; pEPO-treated in gray).

1066 (D) Schematic representation of intracellular recordings from CA1 pyramidal cells (PC)
1067 of electrically evoked AMPA- and NMDA-mediated responses.

1068 (E) Panel depicts the quantification of failure rate of evoked responses for vehicle-
1069 (n=9) and pEPO-treated (n=8) cells. Individual experiments are plotted with a line
1070 connecting failure rate values at -60 and $+40$ mV holding potentials. Histograms
1071 represent mean \pm s.e.m. for each dataset. Failure rate is calculated from 50 sweeps per
1072 cell. *n.s.* not significant (paired t-test)

1073 (F-G) Example tracings and scatter plot of evoked EPSCs from one vehicle- (F) and
1074 one pEPO-treated (G) neuron at -60 and $+40$ mV holding potentials. Failed events were
1075 assigned an amplitude of zero for ease of visualization.

1076

1077 **Figure 5. Prolonged EPO (pEPO) downregulates afferent-evoked synaptic**
1078 **transmission.**

1079 (A) Input/Output curves from vehicle-, AD-, pEPO- and both AD/pEPO-treated slices
1080 (n=7-9 per condition), where the fEPSP slope is plotted as a function of stimulus

1081 intensity. Data represented as mean \pm s.e.m. with non-linear fittings. *Right inset:*
1082 histogram depicting the top parameter of the fitted curves in (A) for each condition \pm
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 μ A
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 \pm 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 \pm 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 \pm s.e.m. * p <0.05, ** p <0.01, **** p <0.0001,
1109 as compared to vehicle; ^p 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-AMPA) 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 μ M) 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 \pm 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 \pm s.e.m. ** p <0.01, *** p <0.001, as compared to
1126 vehicle; ^{φφ} p <0.01, ^{φφφ} p <0.001, as compared to AD (one-way ANOVA with
1127 Bonferroni's correction).

1128

1129