Revisiting adult neurogenesis and the role of erythropoietin for neuronal and oligodendroglial differentiation in the hippocampus

Imam Hassouna[1]*, Christoph Ott[1]*, Liane Dahm[1]*, Nils Offen[2], Richard A. Neher[3],

Miso Mitkovski[4], Daniela Winkler[1], Swetlana Sperling[1], Lucia Fries[2], Sandra Goebbels[5],

Ingrid C. Vreja[6,7], Nora Hagemeyer[1], Marcus Dittrich[8], Maria Florencia Rossetti[1],

Katharina Kröhnert[6], Kathrin Hannke[1], Susann Boretius[9], Andre Zeug[10], Carmen Höschen[11],

Thomas Dandekar[8], Ekrem Dere[1], Erwin Neher[12,13], Silvio O. Rizzoli[6,13],

Anna-Leena Sirén[2], Klaus-Armin Nave[5,13]§, and Hannelore Ehrenreich[1,13]§

*These authors contributed equally to this work.

[1]Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen,
[2]Department of Neurosurgery, University of Würzburg,
[3]Evolutionary Dynamics & Biophysics, Max Planck Institute for Developmental Biology, Tübingen,
[4]Light Microscopy Facility, Max Planck Institute of Experimental Medicine, Göttingen,
[5] Department of Neurogenetics, Max Planck Institute of Experimental Medicine, Göttingen,
[6]Department of Neuro- and Sensory Physiology, University Medical Center Göttingen,
[7]International Max Planck Research School Molecular Biology, Göttingen,
[8]Department of Bioinformatics, Biocenter, University of Würzburg,
[9]Department of Diagnostic Radiology, Christian-Albrechts-Universität, Kiel,
[10]Cellular Neurophysiology, Hannover Medical School, Hannover,
[11]Department of Ecology and Ecosystem Management, Lehrstuhl für Bodenkunde,
Technische Universität München, Freising-Weihenstephan,
[12]Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, Göttingen,
[13]DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen,
GERMANY

Supplementary Information

Supplementary Figures 1-5, provided as separate files

Supplementary Figure Legends 1-5

Supplementary Tables 1-5

Supplementary Figure 1: Cresyl violet staining of a coronal hippocampus (HC) section. Shown is a coronal section at approximately bregma –1.8 mm, stained with cresyl violet. Insert in the upper left presents a magnification of the pyramidal layer. Insert in the upper right gives a schematic view of the hippocampus (modified from Franklin and Paxinos, 1997). Corpus callosum (CC), cornu ammonis 1 & 3 (CA1 & CA3), and dentate gyrus (DG) are indicated. The pyramidal layer (Py) within CA1 and CA3 is highlighted in red to show where pyramidal neurons (as well as NeuN+/BrdU+ double-labeled neurons) were counted. CA1, CA3 and DG are framed in yellow to demonstrate where BrdU, TUNEL, Dcx, Pax6, Olig1, PDGFRα and NG2 were stereologically quantified.

Supplementary Figure 2: EPOR expression in OPC. (A) EPOR expression in NG2-cells of NG2-Cre-ERT2:R26R-td-tomato-mEGFP mouse brains at postnatal day 31. Lower subpanels show magnification of all channels of the white square area denoted in the overview. **(B)** Western Blot showing specificity of the used EPOR antibody *(Ott C, Martens H, Hassouna I, Oliveira B, Erck C, Zafeiriou MP et al. Widespread expression of erythropoietin receptor in brain and its induction by injury. Mol Med 2015).* **(C)** qPCR analysis of oligodendrocyte lineage genes and *EPOR* in primary mouse forebrain OPC *in vitro* cultured in serum-free differentiation medium. All qPCR data are normalized to *Hprt* (housekeeper). (n=5 for all genes at 4h and 18h time points and n=4 at 32h, mean±SEM, one-way ANOVA); **P<0.01, ***P<0.001.

Supplementary Figure 3: EPO effects on neurogenesis in dentate gyrus (DG) and subventricular zone (SVZ). All data are based on bilateral counting. **(A-F)** Proliferation (BrdU+ cells, placebo n=9 and EPO n=10 for DG; placebo n=8 and EPO n=9 for SVZ), apoptosis (Tunel+ cells, placebo n=9 and EPO n=10 for DG; placebo and EPO n=10 for SVZ) and differentiation (Dcx+ cells) at 1 week (w1, placebo n=10 and EPO n=9 for both DG and SVZ) and 4 weeks (w4, placebo n=5 and EPO n=6 for BrdU+ cells; placebo n=7 and EPO n=9 in DG, placebo n=9 and EPO n=10 in SVZ for tunel+ cells; placebo n=8 and EPO n=10 for Dcx+ cells for both DG and SVZ) after start of EPO/placebo treatment. **(G-I)** Confocal analysis of BrdU and NeuN double-positive cells (n=4 for both groups; BrdU+, red; NeuN+, green) and presentation of a sample picture in 2 magnifications. All bar graphs are shown as mean±SEM; all analyses unpaired, two-tailed *t*-tests; *P<0.05, **P<0.01.

Supplementary Figure 4: EPO effects on quaternary E14 forebrain neurospheres cultured under proliferating and differentiating conditions: (A,B) Quaternary E14 forebrain neurospheres cultured under proliferating conditions: **(A)** Experimental design. **(B)** qPCR analysis of EPOR expression (n=3) and EPOR functionality testing by Western blot analysis of pMAPK/MAPK (n=6 per group, paired two-tailed t-test) 10min after EPO/placebo at 24h in culture. **(C-H)** Quaternary E14 forebrain neurospheres cultured under differentiating conditions: **(C)** Experimental design. **(D)** qPCR analysis of EPOR expression (n=3) and EPOR functionality testing by Western blot analysis of pMAPK/MAPK (n=7 per group, paired one-tailed t-test) 10min after EPO/placebo after 24h in culture, respectively. **(E)** qPCR results showing relative mRNA expression under placebo conditions of the differentiation markers Sox9, ND1, Dcx, MAP2, GFAP and S100B at 24h, 96h and 168h expressed as percent of the respective 0.3h values (Sox9: 0.3h n=6, 24h n=10, 96h n=9, 168h n=9; ND1: 0.3h n=6, 24h n=10, 96h n=10, 168h n=9; Dcx: 0.3h n=6, 24h n=10, 96h n=10, 168h n=9; MAP2: 0.3h n=6, 24h n=10, 96h n=10, 168h n=9; GFAP: 0.3h n=6, 24h n=10, 96h n=10, 168h n=9; S100B 0.3h n=6, 24h n=6, 96h n=6, 168h n=6). **(F)** Culture composition showing Tuj1+ and GFAP+ cells in percent of total cell number (DAPI) (n=4 per group). **(G)** Cell death analyzed with Trypan blue (n=4 per group, paired two-tailed t-test). **(H)** Cell number (DAPI counts) (n=4 per group, paired two-tailed t-test). All bar graphs are shown as mean±SEM; *$P<0.05$.

Supplementary Figure 5: EPO effects on differentiation of E17 hippocampal cultures (E17 HCC). (A) Experimental design. **(B)** qPCR analysis of EPOR expression (n=4 per group) and EPOR functionality testing by Western blot analysis of pMAPK/MAPK (n=4 per group, paired two-tailed t-test) 10min after EPO/placebo at the seeding time point and at 24h in culture, respectively. **(C)** qPCR results showing relative mRNA expression under placebo conditions of the differentiation markers Sox9, ND1, Dcx, MAP2, GFAP and S100B at 0.5, 1, 3, 6, 12 and 24h expressed as percent of the respective 0.25h values (Sox9: 0.25h n=10, 0.5h n=7, 1h n=8, 3h n=8, 6h n=8, 12h n=4, 24h n=6; ND1: 0.25h n=9, 0.5h n=5, 1h n=7, 3h n=7, 6h n=7, 12h n=4, 24h n=6; Dcx: 0.25h n=9, 0.5h n=8, 1h n=6, 3h n=6, 6h n=7, 12h n=6, 24h n=9; MAP2: 0.25h n=14, 0.5h n=9, 1h n=10, 3h n=8, 6h n=8, 12h n=8, 24h n=12; GFAP: 0.25h n=7, 0.5h n=7, 1h n=6, 3h n=7, 6h n=7, 12h n=8, 24h n=8; S100B: 0.25h n=8, 0.5h n=8, 1h n=5, 3h n=7, 6h n=7, 12h n=7, 24h n=6). **(D,E)** qPCR results of the early neural markers Sox9 (0.25h n=10, 0.5h n=7, 1h n=8, 3h n=8, 6h n=8, 12h n=4, 24h n=6; paired two-tailed t-test per time point) and ND1 (0.25h n=9, 0.5h n=5, 1h n=7, 3h n=7, 6h n=7, 12h n=4, 24h n=9; paired two-tailed t-test per time point) after

EPO, expressed in % placebo **(F)** Dcx/MAP2 ratio after EPO, expressed in % placebo (0.25h n=7, 0.5h n=7, 1h n=4, 3h n=6, 6h n=5, 12h n=4, 24h n=4; paired two-tailed *t*-test per time point). **(G)** Dcx/MAP2 ratio based on immunocytochemical analysis of integrated density (n=5 per group, paired two-tailed *t*-test per time point)). **(H)** Confocal picture illustrating the distribution of Dcx and MAP2 staining. **(I,J)** qPCR results of GFAP (0.25h n=7, 0.5h n=7, 1h n=6, 3h n=6, 6h n=7, 12h n=7, 24h n=7; paired two-tailed *t*-test per time point) and S100B (0.25h n=8, 0.5h n=8, 1h n=5, 3h n=7, 6h n=7, 12h n=7, 24h n=5; paired two-tailed *t*-test per time point) after EPO, expressed in % placebo. **(K)** Culture composition showing Dcx+ and GFAP+ cells in percent of total cell number (DAPI) (n=9 per group), P=Placebo, E=EPO. **(L,M)** Cell death and cell number (n=5 for both groups and readouts, paired two-tailed *t*-test per time point) analyzed with DAPI. All bar graphs are shown as mean±SEM; *P<0.05, **P<0.01, ***P<0.001 (paired *t*-test). Compare Supplementary Tables 4 and 5.

Supplementary Table 1
Number of pyramidal neurons in the hippocampus 4 weeks after start of EPO or placebo treatment: Given are numbers obtained upon direct counting ($\sum Q^-$; raw data) and the final numbers according to the stereological calculation (N).

Treatment	Mouse ID (internal)	Pyramidal neurons (#)			
		CA1		CA3	
		$\sum Q^-$	N	$\sum Q^-$	N
PL (1)	CM-2	966	115920	871	104520
	CM-3	947	113640	921	110520
	CN-1	956	114720	926	111120
	CN-4	956	114720	-	-
	CO-1	778	93360	778	93360
	CO-2	834	100080	908	108960
	CO-4	860	103200	874	104880
	CP-1	943	113160	802	96240
	CP-2	977	117240	698	83760
PL (2)	B1*	839	100680	607	72840
	B3*	806	96720	754	90480
	B4*	833	99960	682	81840
	D1*	948	113760	824	98880
	D2*	874	104880	721	86520
	D4*	876	105120	759	91080
	E3*	809	97080	774	92880
	E4*	1003	120360	673	80760
Mean		**894.4**	**107329.41**	**785.8**	**94290**
±SEM		±17.19	±2063	±24.06	±2887
EPO (1)	CM-1	1122	134640	1118	134160
	CM-4	1041	124920	847	101640
	CM-5	1117	134040	952	114240
	CN-3	1076	129120	936	112320
	CN-5	1026	123120	1009	121080
	CO-3	1157	138840	1219	146280
	CO-5	959	115080	844	101280
	CP-3	1012	121440	1114	133680
	CP-4	934	112080	796	95520
	CP-5	1126	135120	981	117720
EPO (2)	A5*	872	104640	752	90240
	B2*	880	105600	792	95040
	B5*	958	114960	1093	131160
	C1*	1064	127680	876	105120
	D3*	915	109800	864	103680
	D5*	889	106680	676	81120
	E1*	1192	143040	1002	120240
	E2*	-	-	667	80040
Mean		**1020**	**122400**	**918.8**	**110253.33**
±SEM		± 24.98	± 2997	± 36.85	±4422

*mice injected with BrdU (cohort2)

The data are calculated with the stereological formula: $$N = \sum Q^- \cdot \frac{1}{\text{ssf}} \cdot \frac{1}{\text{asf}} \cdot \frac{1}{\text{tsf}}$$

The total number of pyramidal neurons (N) is calculated by the count of pyramidal neurons ($\sum Q^-$), multiplied with the section sampling fraction (ssf), the area sampling fraction (asf) and the thickness sampling fraction (tsf). Counting was performed by an investigator unaware of group assignment of the mice ('blinded'). Two independent cohorts of mice were examined, with only the second cohort receiving BrdU injections. Cohort 1: Placebo group 1 (PL (1)) and EPO group 1 (EPO (1)). Cohort 2: Placebo group 2 (PL (2)) and EPO group 2 (EPO (2)). Underlined are the mean values presented in Figure 1B.

Supplementary Table 2

qPCR results (mean±SEM) of mRNA expression in differentiating neurospheres

(Mean values are presented in Supplementary Figure 4E)

	24h	96h	168h
Sox9	-45.68±10.72	-49.78±5.21	-48.66±4.19
ND1	17.66±28.94	274.00±96.06	99.77±24.44
Dcx	28.21±24.87	251.10±45.40	71.24±9.71
MAP2	83.78±10.40	86.17±15.71	69.86±12.51
GFAP	5.17±16.93	57.61±40.92	69.51±47.17
S100B	169.30±38.93	319.20±56.94	223.30±37.15

Data calculated according to the formula: 100*(placebo(t) – mean placebo(t=0.3)) / mean placebo(t=0.3)

Supplementary Table 3

qPCR results (mean±SEM) of mRNA expression in differentiating neurospheres after EPO, expressed as % placebo

(Data are presented in Figure 5D,E,F,G,H)

	0.3h	24h	96h	168h
Sox9	23.63*±7.34	-23.26**±3.74	-31.90±12.59	-15.77***±2.08
ND1	5.96±9.70	-17.83**±3.00	-42.67*±10.83	-27.21*±10.18
Dcx/MAP2	4.41±7.26	12.59±7.01	-27.02*±11.67	-19.97±8.21
GFAP	-2.97±3.44	25.33**±4.89	42.71**±4.63	16.02±39.77
S100B	2.82±5.60	-7.49±3.51	17.03±8.24	11.58±6.07

*P≤0.05, **P≤0.01, ***P≤0.001 (paired *t*-test)
Data calculated according to the formula: 100*(EPO(t)-placebo(t)) / placebo(t)

Supplementary Table 4

qPCR results (mean±SEM) of mRNA expression in E17 HCC

(Mean values are presented in Supplementary Figure 5C)

	0.5h	1h	3h	6h	12h	24h
Sox9	-13.91±30.32	92.21±38.56	46.18±32.76	-36.27±8.25	-54.63±5.87	-64.10±6.55
ND1	-12.74±17.11	-32.58±8.43	-4.71±7.55	-11.95±9.91	11.16±18.59	25.63±13.35
Dcx	-31.42±30.14	-19.69±39.20	-25.57±21.93	-23.15±16.01	-26.01±24.53	-18.14±10.67
MAP2	-0.32±11.81	3.20±10.05	-17.07±7.73	-34.21±6.19	-12.89±7.05	-28.10±14.34
GFAP	21.04±12.76	68.95±19.64	129.80±74.05	105.70±21.57	236.90±30.23	240.90±65.48
S100B	-13.90±22.52	-27.33±14.51	-39.79±10.02	-40.14±10.82	17.74±10.35	247.90±51.03

Data calculated according to the formula: 100*(placebo(t) – mean placebo(t=0.3)) / mean placebo(t=0.3)

Supplementary Table 5

qPCR results (mean±SEM) of mRNA expression in E17 HCC after EPO, expressed as % placebo

(Data are presented in Supplementary Figure 5D,E,F,I,J)

	0.25h	0.5h	1h	3h	6h	12h	24h
Sox9	37.69*±12.39	31.71±19.25	41.02±23.27	-1.80±8.04	8.91±17.08	7.10±9.59	5.20±9.42
ND1	1.02±9.38	-13.44±4.88	-13.77*±4.86	-9.79±6.63	-6.76±7.10	-14.55±12.95	-3.73±11.69
Dcx/MAP2	21.90±16.21	6.20±9.46	30.62±10.67	-0.92±8.43	-15.13*±4.98	0.26±11.45	0.51±6.42
GFAP	10.90±11.77	-3.51±4.97	-20.42*±7.01	-8.69±8.66	0.76±13.60	-8.59±9.46	-2,34±5,97
S100B	-24.19±11.37	-24.81*±8.93	-30.59**±4.98	-4.04±15.22	3.53±9.44	-0.70±7.35	-10.07±6.11

*P≤0.05, **P≤0.01, ***P≤0.001 (paired *t*-test)
Data calculated according to the formula: 100*(EPO(t)-placebo(t)) / placebo(t)