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Spreading depression and focal venous cerebral ischemia enhance cortical neurogenesis

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Abstract
Endogenous neurogenesis can arise from a variety of physiological stimuli including exercise, learning, or "enriched environment" as well as pathological conditions such as ischemia, epilepsy or cortical spreading depression. Whether all these conditions use a common trigger to set off endogenous neurogenesis is yet unclear. We hypothesized that cortical spreading depression (CSD) induces neurogenesis in the cerebral cortex and dentate gyrus after cerebral venous ischemia. Forty-two Wistar rats alternatively underwent sham operation (Sham), induction of ten CSDs or venous ischemia provoked *via* occlusion of two adjacent superficial cortical vein followed by ten induced CSDs (CSD + 2-VO). As an additional control, 15 naïve rats received no intervention except 5-bromo-2′-deoxyuridine (BrdU) treatment for 7 days. Sagittal brain slices (40 μm thick) were co-stained for BrdU and doublecortin (DCX; new immature neuronal cells) on day 9 or NeuN (new mature neuronal cells) on day 28. On day 9 after sham operation, cell proliferation and neurogenesis occurred in the cortex in rats. The sole induction of CSD had no effect. But on days 9 and 28, more proliferating cells and newly formed neurons in the ipsilateral cortex were observed in rats subjected to CSD + 2VO than in rats subjected to sham operation. On days 9 and 28, cell proliferation and neurogenesis in the ipsilateral dentate gyrus was increased in sham-operated rats than in naïve rats. Our data supports the hypothesis that induced cortical neurogenesis after CSD + 2-VO is a direct effect of ischemia, rather than of CSD alone.

Key Words: *nerve regeneration; cortical spreading depression; two-vein occlusion; adult neurogenesis; stem cells; cerebral cortex; neural precursor cells; neuron; penumbra; neural regeneration*

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Introduction
The dogma of the brain's inability to replace lost neuronal cells has been undeniably refuted. Today at least two regions of the adult brain are known to retain the ability to continuously produce neuronal cells throughout the life. These "neurogenic" regions are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal formation (Lois and Alvarez-Buylla, 1994; Cooper-Kuhn and Kuhn, 2002; Carleton et al., 2003). There is also growing evidence suggesting the presence of neural stem cells in other "non-neurogenic" regions of the brain to include the cerebral cortex, caudate nucleus, and the striatum (Kaplan, 1981; Gould et al., 1999b; Bernier et al., 2002). However, unlike the SVZ and SGZ, comparatively very low levels of neurogenesis exist in these latter regions under physiological conditions.

Transient cortical spreading depression (CSD) is a wave of sustained neuronal depolarization moving through intact brain tissues at the speed of 2–5 mm/min. This phenomenon first described by Leão (1944) can be triggered by increased extracellular potassium following electrical, mechanical or chemical stimuli and is associated with "periinfarct depolarizations." In clinical and experimental settings, CSD can be induced through the application of potassium chloride (KCl) into the brain. The effects of CSD are presumed to be favorable to brain cells and neuroprotective due to its preconditioning effects, upregulation of trophic substances such as brain-derived neurotrophic factor (BDNF) and protection against subsequent focal brain ischemia (Kokaia, 1993; Matsushima et al., 1998; Yanamoto et al., 2005). However, other studies also suggest that CSD due to increased adenosine triphosphate (ATP) consumption (negative energy balance) and 'inverse' vascular coupling may well contribute to increased tissue hypoxia as well as secondary injury in stroke and traumatic brain injury (Nedergaard and Hansen, 1988; Takano et al., 2007).

Based on our previous findings on the pathophysiological processes involved in cerebral ischemia using a rat two-vein occlusion model (Nakase et al., 1996, 1997; Heimann et al., 2003), cerebral venous occlusion as a result of relative low flow area compared to the ischemic core causes a slowly developing tissue infarction that leads to local tissue demise (Kempski et al., 1999). Unlike the arterial occlusion, the penumbra area after venous occlusion is sustained over a longer period of time. In combination with CSD, the damaging effect on tissue is more severe (Otsuka et al., 2000). Both CSD and cerebral ischemia individually have been reported to augment the response of adult stem cells *in vivo* (Tamura et al., 2004; Yanamoto et al., 2005). The aim of this study is therefore to clarify whether CSD alone versus CSD in combination with cerebral venous ischemia is able to trigger neurogenesis in the cerebral cortex and dentate gyrus (DG).

Materials and Methods

Animals

This study is approved by the Landesuntersuchungsamt Rheinland-Pfalz (approval No. AZ: 177-07/051-16), and was performed in accordance with the German animal protection law. All efforts were made to minimize the number and suffering of animals used in this experiment.

Forty-two male Wistar rats (7–9 weeks old, Charles Rivers, Germany), weighing 315–359 g, were randomized into three groups: Sham (n = 14), CSD (n = 14), and CSD plus two-vein occlusion (CSD + 2-VO; n = 14). A 2-VO group without additional CSD induction was consciously abandoned because of its important variability of spontaneously occurring CSD which go along with altering infarction volume causing problems in statistical and interpretation of pathophysiological pathways (Otsuka et al., 2000). Seven animals in each experimental group were observed for either 9 or 28 days according to their survival time. In order to assess the rate of neurogenesis under physiological conditions two additional groups of naïve animals received 7 days of BrdU treatment and were sacrificed after 9 (n = 7) and 28 days (n = 8), respectively. Animals were housed in individual cages and allowed free access to food and water *ad libitum* prior to and after surgery.

Animal preparation

Animals were premedicated with 1 mg atropine sulfate and anesthesia was performed by intraperitoneal injection of chloral hydrate (36 mg/100 g body weight). Rats were intubated and mechanically ventilated with 30% oxygen under controlled end respiratory PCO_2 (Artema MM206C; Heyer, Sweden) using a rodent ventilator (Model 683; Harvard Apparatus, MA, USA). Rectal temperature was maintained at 37°C *via* a feedback-controlled heating pad (Harvard Apparatus, MA, USA). The tail artery was cannulated using a polyethylene catheter (outer diameter 0.96 mm) to measure arterial blood pressure (MABP; Gould transducer 134615-50), and to monitor blood gases, electrolytes, glucose, hematocrit and pH levels (ABL System 612/EML6, Radiometer, Denmark) during operation. The femoral vein was catheterized for drug administration. Rats were placed in a stereotactic frame (Stoelting, Wood Dale, IL, USA) a left cranial window was drilled under an operating microscope (OP-Microscope; Zeiss, Wetzlar, Germany) to access the brain. To avoid thermal injury, the tip of the drill was continuously cooled with physiological saline during craniotomy. As described previously (Nakase et al., 1996, 1997), regional cerebral blood flow (rCBF) was measured using laser Doppler (LD) scanning (Model BPM 403a; Vasomedics, St. Paul, MN, USA) with a 0.8-mm needle probe. Flow is expressed in LD units. A micropipette (GB150F10, Science Products GmbH Hofheim, Germany pulled by Micropipette Puller P-87, Navato, CA, USA) was inserted into the cerebral cortex for application of KCl. Baseline values were taken 90 minutes after insertion, before initiation of venous ischemia.

Cortical vein occlusion by photochemical thrombosis

Two adjacent superficial cortical veins connecting into the superior sagittal sinus were occluded using Rose Bengal dye (Sigma Chemical Co., St. Louis, MO, USA) in combination with fiberoptic illumination (100-W mercury lamp [6,500–7,500 lx, 540 nm]) connected to a 200-µm fiber. Only animals which presented similar anatomy (*i.e.*, two prominent adjacent cortical veins connecting into the superior sagittal sinus) were used in this study. The diameter of occluded veins was approximately 80–100 µm. Rose Bengal at the dose of 50 mg/kg was slowly injected intra¬venously; target veins were then selectively illuminated using a micromanipulator-assisted fiber-optic light guide with care taken to avoid illumination of nearby tissue and other blood vessels. Target veins were illuminated for 10 minutes. Prior to the illumination of the second target vein, half of the initial Rose Bengal dosage was additionally administered intravenously before the illumination was undertaken. Occlusion of veins was confirmed with the laser Doppler system detecting acutely decreased blood flow at the site of the veins and its surrounding tissues.

Induction of cortical spreading depression

All animals randomly assigned to receive CSD or CSD + 2-VO occlusion were subjected to 10 cortical spreading depressions induced via the intracerebral application of 2 µL of a 150 mM potassium chloride (KCl) solution by a glass micropipette and a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden), with 7-minute intervals between each injection, and administration procedures lasting for 70 minutes overall. Rats in the CSD group were subjected to KCl administration without previous 2-VO, while rats in the combined group received 2-VO before KCl administration. Tissue impedance as an indicator for cell swelling during CSD was measured continuously using two impedance electrodes (0.4–0.5 mm depth, 3 mm from occluded vein, stainless steel wires, outside diameter 0.5 mm) covered with polyurethan insulating sheath - except for its exposed tips (outer diameter 0.3 mm). Impedance measurements were recorded at 1 kHz (1 µA, 10 mV, bias-free) continuously throughout the course of the experiment using a precision LCR monitor (4284A; Hewlett-Packard, Hewlett-Packard, Salt Lake City, UT, USA). At the 1 kHz frequency, alternating currents spread throughout the extracellular space and impedance increases when extracellular space shrinks, *i.e.*, cells swell (Otsuka et al., 2000). Animals randomly assigned to sham groups underwent surgical procedures with the placement of electrodes without the 2-VO or the induction of CSD.

BrdU injection and immunofluorescence analysis

After completion of surgery, skin lesions were sutured with 4-O silk and animals were returned to their individual cages and granted ad libitum access to food and water. To gain access to dividing cells, the thymidine analogue BrdU was used to label dividing cells. First *in-vivo* labeling with BrdU was started 24 hours after operation. Seven BrdU injections (100

mg/kg body weight, i.p., per day for 7 days) were administered. Euthanasia was performed on the 9th or 28th postoperative days in deep anesthesia (chloral hydrate 72 mg/100 g) and animals were perfused transcardially with combined physiological saline and heparin (20,000 IE/L) followed by 4% paraformaldehyde in 0.2 M phosphate buffer solution (pH 7.4). Brains were carefully harvested, post-fixed 24 hours in paraformaldehyde-phosphate buffer, and then stored in 30% sucrose solution (400 mL 0.1 M PO_4 and 150 g Saccharose) at 4°C until microtomy. 40 μm-thick sagittal sections were collected using a sledge microtome.

Immunohistochemistry was performed to count all newly formed cells in the DG in free floating brain sections. Slices were stained with a monoclonal mouse-anti-BrdU (Roche Diagnostics Cooperation; 1:500) and a biotinylated donkey anti-mouse IgG. Fluorescence microscopy was used to calculate the number of double-labeled BrdU- and DCX- or NeuN-positive newly formed cells (neurons) in relation to the total amount of newly formed cells in the DG as previously described (Engelhard et al., 2007).

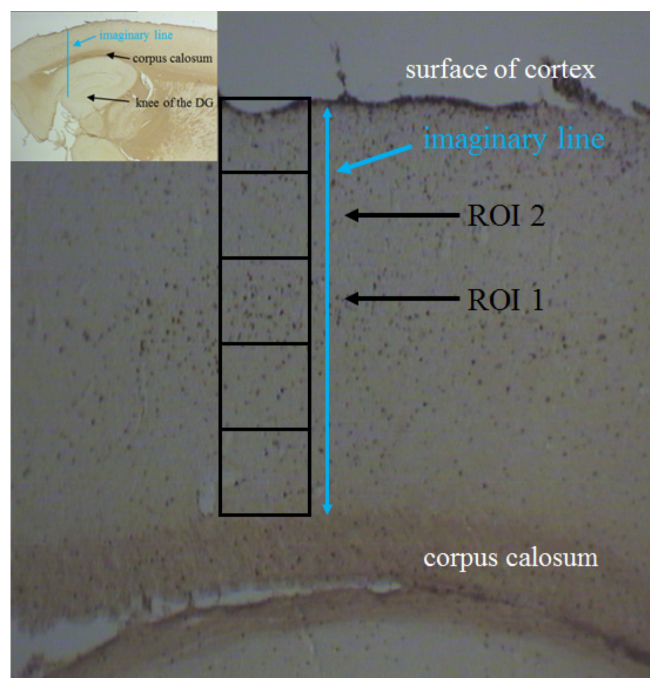

Figure 1 Graphic description of ROI placement in brain sections.
Five windows (120×160 μm^2 each) were placed in an imaginary line (600 μm) from the knee of the dentate gyrus to the surface of the cortex that ROI I was centered and ROI II started just above. ROI: Region of interest.

For immunofluorescence, free floating brain sections were stained with rat-anti-BrdU primary antibody (1:500; rat anti-BrdU, Oxford Biotechnology) and donkey-anti-rat secondary antibody (1:500; Fluorescein AffiniPure Donkey Anti-Rat IgG, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). The neuronal markers used were DCX for the 9 days group and NeuN for the 28 days groups). Primary and secondary antibodies for DCX were goat-anti-DCX (1:500; sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and donkey-anti-goat (1:500; Rhodamine Red™-X AffiniPure Donkey Anti-Mouse IgG, Jackson ImmunoResearch Laboratories), respectively. Primary and secondary antibodies for neuron-specific nuclear protein or NeuN were mouse-anti-NeuN (1:250; MAB377, Chemicon International, Inc., Billerica, MA, USA) and donkey-anti-mouse (1:500; Rhodamine Red™-X AffiniPure Donkey Anti-Mouse IgG, Jackson ImmunoResearch Laboratories; see Engelhard et al., 2007). Unlike the DG, the immunofluorescent-labelled cells were counted in the cortex only within the predefined regions of interest (ROI I and II). They were placed in the middle of the cortex between the corpus callosum and the surface of the cortex (**Figure 1**), *i.e.*, in the area where the 2-VO was induced. The frame dimension of one ROI was set to 220 μm × 160 μm (40× magnifications). Two ROIs per section and three sections per hemisphere were analyzed for each animal to assess differences with possible references to migrating cells from "neurogenic" zones towards the cerebral cortex, or perhaps activated resident neural stem cells (NSCs) in the cerebral cortex. The numbers of cells gathered in the examined ROI for respective hemispheres are normalized to mm^2.

Statistical analysis

Statistical analysis was performed using SigmaStat® 3.5

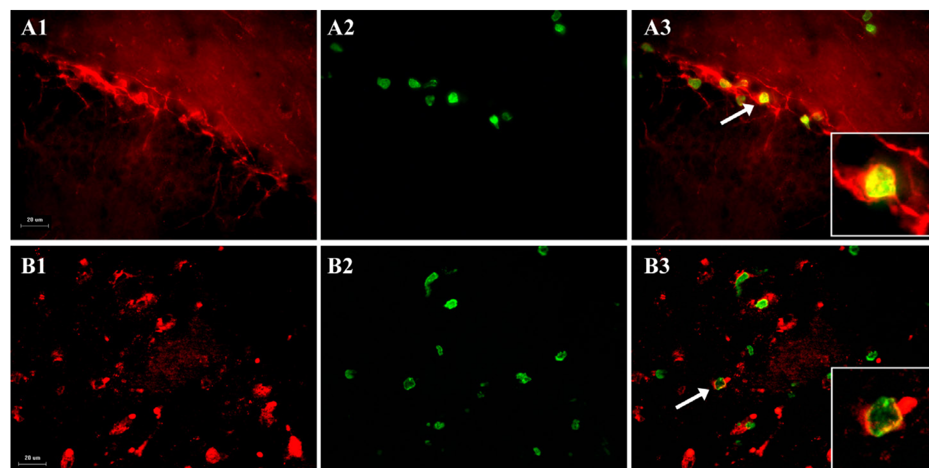

Figure 2 Typical micrographs from dentate gyrus (A) and cortex (B) showing doublecortin-positive cells on day 9 (A1) in the dentate gyrus and neuronal nuclei (NeuN)-positive cells (red) on day 28 (B1) in the parietal cortex of rats with cortical spreading depression plus cerebral venous ischemia.
Newly formed cells are 5-bromo-2′-deoxyuridine (BrdU)-labeled (green, A2; B2). Merged micrographs identify newly formed neurons by co-localization of BrdU and doublecortin on day 9 in the dentate gyrus (A3) and co-localization of BrdU and NeuN on day 28 in the cortex (B3). Scales: 20 μm, arrows point to a typical double-labeled cell which is magnified in the inlet.

Table 1 Physiological parameters during baseline conditions of rats with CSD + 2-VO

Group	Weight (g)	MABP (mmHg)	rCBF (LDU)	Rectal temperature (°C)	Blood gas analysis			
					pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	Htc (%)
Naïve								
9-day	343.00±2.29							
28-day	339.00±1.91							
Sham								
9-day	339.57±4.72	76.40±3.0	46.11±5.3	37.16±0.1	7.39±0.026	41.87±2.1	119.34±20.3	44.31±2.3
28-day	326.00±4.32	78.76±2.8	49.38±8.4	36.88±0.2	7.42±0.039	41.30±3.3	129.30±11.7	43.88±1.3
CSD								
9-day	344.29±1.87	85.90±4.9	56.96±8.1	37.16±0.2	7.41±0.034	40.34±3.0	118.73±12.3	44.66±2.6
28-day	333.63±3.34	85.45±3.5	64.52±7.1	37.08±0.1	7.40±0.046	42.46±1.7	128.28±10.4	43.44±2.8
CSD+2-VO								
9-day	338.29±4.70	87.06±3.7	45.20±7.5	37.37±0.1	7.40±0.037	41.20±1.7	131.56±10.0	43.91±2.8
28-day	338.14±5.36	91.09±4.1	66.28±7.0	36.74±0.1	7.42±0.028	41.00±2.3	132.70±14.7	44.54±1.6

Data are presented as the mean ± SEM. CSD: Cortical spreading depression; 2-VO: two-vein occlusion; MABP: mean arterial blood pressure; rCBF: regional cerebral blood flow; LDU: laser Doppler units; $PaCO_2$: arterial carbon dioxide tension; PaO_2: arterial oxygen tension; Htc: hematocrit.

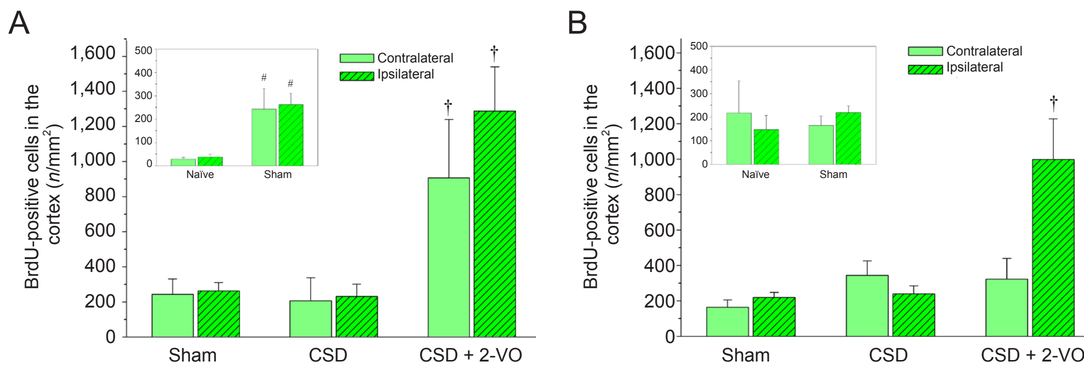

Figure 3 Cell proliferation on days 9 (A) and 28 (B) in the cortex of rats with cortical spreading depression (CSD) plus two-vein oclusion (2-VO). 5-Bromo-2′-deoxyuridine (BrdU)-labeled cells are bilaterally increased in sham-operated animals on day 9 (inlet: #*P* < 0.05, *vs*. naïve animals; Mann-Whitney Rank Sum Test). But only CSD combined with 2-VO induced a significant higher cell proliferation compared to CSD alone or sham at both evaluated time points (†*P* < 0.05; Kruskal-Wallis one-way analysis of variance by ranks). Data are presented as the mean ± SEM. The number of experiments generally is 7. *N* = 6 was only in the CSD + 2VO group on day 9 and *n* = 8 in naïve and CSD + 2-VO groups on day 28.

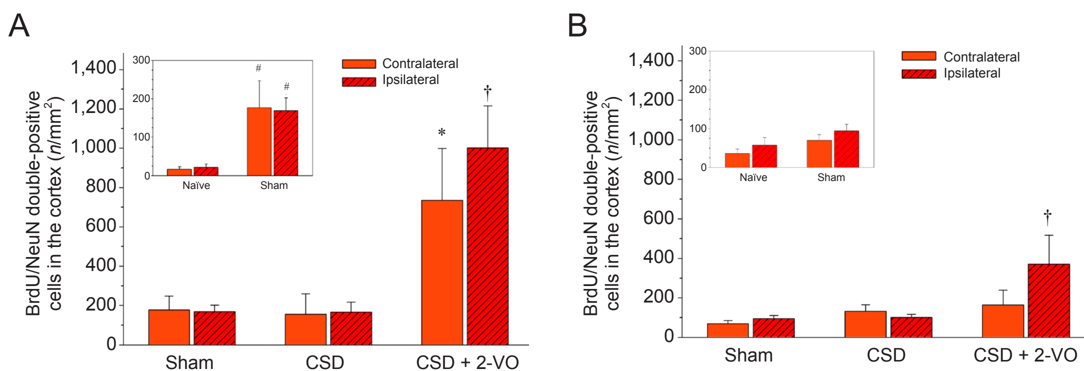

Figure 4 Neurogenesis on days 9 (A) and 28 (B) in the cortex of rats with cortical spreading depression (CSD) plus two-vein occlusion (2-VO). Newly formed neurons were induced after CSD combined with 2-VO on days 9 and 28 in the ipsilateral hemisphere (†*P* < 0.05, *vs*. sham and CSD groups; Kruskal-Wallis one-way analysis of variance by ranks). Interestingly, on day 9, more BrdU/DCX double-labeled cells are identified in sham operated animals compared to naïve animals (A: inlet #*P* < 0.05, *vs*. naïve group; Student's *t*-test) and in the contralateral hemisphere in the CSD + 2-VO group compared to sham group (**P* < 0.05, Kruskal-Wallis one-way analysis of variance by ranks). Data are presented as the mean ± SEM. The number of experiments is 6 in the CSD + 2-VO group and 7 in naïve, sham and CSD groups on day 9. On day 28, there were 7 rats per group in the sham and CSD groups and 8 rats in the naïve and CSD + 2-VO groups.

Table 2 Counts per mm² of newly formed BrdU-positive cells and percentages of co-staining with neuronal markers (DCX for 9 days; NeuN for 28 days) in the cerebral cortex of rats subjected to CSD plus 2-VO

	9 Days (*n* = 27) DCX				28 Days (*n* = 30) NeuN			
	ROI 1		ROI 2		ROI 1		ROI 2	
Group	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Sham								
BrdU⁺	115±39	125±17	129±49	138±32	83±23	107±15	83±19	112±18
DCX⁺ or NeuN⁺	83±31	79±14	95±39	91±21	41±9	41±9	30±8	54±10
CSD								
BrdU⁺	129±93	112±36	79±41	119±36	184±38	114±24	160±46	126±24
DCX⁺ or NeuN⁺	99±74	77±26	57±32	89±28	70±11	47±8	62±23	54±9
CSD + 2-VO								
BrdU⁺	466±186*	661±120*	442±148*	627±144*	160±67	496±104*#†	163±50	503±127*#†
DCX⁺ or NeuN⁺	368±150*	513±103*	368±114	489±119	83±42*	186±66*	82±33*	185±82*
Naïve								
BrdU⁺	16±6	15±7	12±4	23±6	100±65	84±34	117± 72	64±25
DCX⁺ or NeuN⁺	7±5	11±5	10±4	11±5	18±7	34±14	18±6	24±7

Data are expressed as the mean ± SEM. **P* < 0.005, *vs*. sham group; #*P* < 0.005, *vs*. naïve group; †*P* < 0.005, *vs*. CSD group; Student's *t*-test. *n* = 27 and *n* = 30 indicate the total number of experiments performed with the staining techniques at the evaluated time points 9 and 28 days respectively. BrdU: 5-Bromo-2′-deoxyuridine; DCX: doublecortin; CSD: cortical spreading depression; 2-VO: two-vein occlusion.

Table 3 Counts (*n*) of newly formed BrdU-positive cells and double-labeled with neuronal markers DCX for 9 days and NeuN for 28 days in the dentate gyrus

	9 Days (*n* = 27)		28 Days (*n* = 30)	
	DCX		NeuN	
Group	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Sham				
BrdU⁺ cells	510±134	826±99#	244±52	626±119#
DCX⁺ or NeuN⁺ cells	462±129	747±92	187±43	463±93
CSD				
BrdU⁺ cells	595±162	1,443±276	320.83±66	1,020±159
DCX⁺ or NeuN⁺ cells	509±143	1,250±255	237±50	747±125
CSD + TVO				
BrdU⁺ cells	693±263	1,527±288	590±130*	1,763±565
DCX⁺ or NeuN⁺ cells	485±183	1,100±87	441±108	1,252±417
Naïve				
BrdU⁺ cells	269±42	263±63	191±27	195±45
DCX⁺ or NeuN⁺ cells	236±33	209±50	151±21	153±37

Data are expressed as the mean ± SEM. **P* < 0.005 *vs*. sham group; #*P* < 0.005, *vs*. naïve group; Student's *t*-test. *n* = 27 and *n* = 30 indicate the total number of experiments performed with the staining techniques at the evaluated time points 9 and 28 days respectively. BrdU: 5-Bromo-2′-deoxyuridine; DCX: doublecortin.

(Jandel Scientific Software, San Jose, CA, USA). Graphs were done with the graphing software SigmaPlot Version 10.0 (Systat Software GmbH, Erkrath, Germany). Data and graphs are presented as the mean ± SEM. Sequential changes between sham, CSD, and CSD + 2-VO groups were statistically evaluated using a Kruskal-Wallis H test (one-way analysis of variance on ranks) followed by Dunn's multiple comparison test for individual group differences. A Student's *t*-test was performed for comparison between sham-operated and naïve animals. If normality test or equal variance test failed, the Mann-Whitney Rank Sum Test was executed. Statistics were performed on a 5% level of significance.

Results

Physiological data

The rats were anesthetized and maintained under baseline conditions in normotensive (mean arterial blood pressure > 70 mmHg) and normocapnic states. All controlled parameters were kept within physiological range without statistical differences observed between groups (not significant: MABP, $PaCO_2$, PaO_2, rectal temperature; **Table 1**). Mean rCBF was measured in the target area between the later occluded veins and varied between 45.2 and 66.3 LDU without any difference in baseline between groups (**Table 1**). Over the total observation period, rCBF declined by 11.3 % in the CSD group and significantly decreased by 68.5% in the CSD + 2-VO groups. There was no significant difference in the counts of measured CSDs between the CSD and CSD + 2-VO groups. There were 16.71 ± 4.8 and 14.13 ± 1.6 counts of CSDs in the CSD 9-day and CSD 28-day groups, respectively, and 19.71 ± 4.0 and 12.86 ± 2.2 counts of CSDs in the CSD + 2-VO 9-day and CSD + 2-VO 28-day groups, respectively.

Immunofluorescence analysis results

Cerebral cortex

Two defined cortical regions of interest (ROI I and ROI II; **Figure 1**) in neurogenic areas and the expected cortical

lesion after 2-VO were determined to investigate changes based on migratory effects. This evaluation did not reveal any difference between the number of newly formed cells or newly formed neurons, neither by spatial nor by temporal pattern (**Table 2**). Thus, both ROIs are summarized and presented together. Immunofluorescence staining (**Figure 2**) demonstrates an increase of BrdU-positive, newborn cells and double labeled $BrdU^+/DCX^+$ newborn neurons in both the ipsilateral and contralateral cortex of sham-operated versus naïve animals only at the early observation time (inlet **Figures 3A** and **4A**). Similarly, the combination of CSD + 2-VO led to a significant increase in the numbers of newly formed cells only on day 9 and correspondently, at a lower level, in number of new neurons in the ipsilateral and contralateral cerebral cortex compared to CSD alone or sham operation (**Figures 3A** and **4A**). In contrast, on day 28, cell proliferation and neurogenesis were identified solely in the ipsilateral cortex in the CSD + 2-VO group (**Figures 3B** and **4B**).

DG

Immunohistological analysis in the DG differentiated sham-operated rats from naïve rats by a significantly higher generation of newly formed cells and newly formed neurons in the ipsilateral hemisphere. Moreover, CSD or CSD combined with 2-VO tended to induce more BrdU/DCX double-stabled cells mainly after 9 days compared to sham operation (**Table 3**). Nonetheless, there was no statistical difference between sham, CSD and CSD + 2-VO groups. Likewise, the number of newly formed cells and newly formed neurons on day 28 was higher in the ipsilateral DG after CSD or CSD + 2-VO induction than in naïve or sham operated rats. In the contralateral hemisphere, the number of newly formed cells and neurogenesis were generally more discrete and was increased after CSD + 2-VO compared to naïve rats (for numbers please see **Table 3**).

Discussion

This study was designed to compare the effects of CSD alone with the combination of CSD and cerebral venous ischemia on neurogenesis in the cerebral cortex and DG. Our results demonstrated that under physiological conditions, spreading depression had no effect on proliferation or neurogenesis in the parietal cortex. This could have been expected since depolarization waves will challenge cerebral blood flow, without causing any damage when CBF and coupling are not hampered by pathophysiological conditions. This evidence is in line with the findings from other groups throughout the world. Nacher et al. (2001) detected DCX expression in different regions of the brain of naïve Sprague-Dawley rats and found only low levels of DCX-positive cells outside of the hippocampus and SVZ/olfactory bulb axis. Likely, other studies implemented on rodents, primates and lagomorphs indicate the generation of new neurons under normal conditions in several classically considered non-neurogenic regions of the mature brain, such as the visual cortex (Kaplan, 1981), striatum (Rakic, 2004; Luzzati et al., 2007), amygdala (Bernier et al., 2002) and neocortex (Gould et al., 1999a). Quantitative studies point toward an extremely low number of mature newly generated neurons. Nonetheless, even low neurogenic activity of neurogenesis under physiological conditions supports the thesis that many regions of the mature brain potentially retain populations of latent progenitor cells.

Proliferation in the cortex occurred in our study only when CSD was combined with focal venous ischemia. Newly formed cells were increased after 9 days in the ipsilateral and contralateral hemispheres. Proliferation on day 28 was less pronounced and happened only in the ipsilateral cortex. The similar pattern was detected in neurogenesis showing newly formed neurons after 9 and 28 days in the parietal cortex in the CSD + 2-VO group only. On day 28, the magnitude was also less pronounced compared to that on day 9. Since CSD alone had no effect, the stimulating trigger for proliferation and formation of newly formed immature neurons has to be induced by ischemic signaling and/or neuronal damage. Furthermore, these findings point out that the effect of CSD depends on the state of the cerebral tissue; in the normal brain, CSD has no pathological consequence to the parenchyma (Nedergaard and Hansen, 1988). Similar to preconditioning when short ischemic episodes before severe injury are able to protect the brain, Yanamoto et al. (2005) have shown that the pretreatment with prolonged KCL-infusion starting 48 hours before focal ischemia in a 3-vessel-occlusion mouse model with partial reperfusion reduced infarction size and had neuroprotective potential. In contrast, an increasing number of spreading depression positively correlates with growing infarct volume in the model of 2-VO (Otsuka et al., 2000) and peri-infarct depolarization increases infarction after middle cerebral artery occlusion (Mies et al., 1993). These phenomena support the theory that CSD aggravates neuronal damage solely "in tissue at risk", *i.e.*, in the penumbra, where energy supply is no longer sufficiently sustained to maintain resting metabolism. CSD may trigger proliferation and neurogenesis in a non-neurogenic zone through "inverse" coupling and neuronal damage after challenging tissue at risk. Likely, Gu et al. (2000) confirmed persistent stimulation of neurogenesis in the DG as well as in the "non-neurogenic" cortical layer IV after CSD and photothrombotic stroke. These reports are in line with the literature which suggests spreading depression to be potentially associated with migraines, seizures, head injury and cerebral ischemic infarction (Hadjikhani et al., 2001; Fabricius et al., 2006), ensuring the potential to stimulate persistent neurogenesis or to produce ectopic new neuron-like cells (Yanamoto, 2005; Xue et al., 2009). Although the mechanism is unknown, non-neurogenic regions of the brain such as cortex are proposed to become more permissive to neurogenic processes in pathological conditions, possibly trying to compensate for the loss of neurons.

Interestingly CSD plus ischemia induced proliferation and neurogenesis in the contralateral hemisphere, which disappeared after 28 days. Similar effects have been reported in the contralateral hemisphere after focal ischemia by middle cerebral artery occlusion (Garbuzova-Davis et

al., 2013; Arango-Dávila et al., 2016) proposing different mechanisms for diaschisis. The loss of excitatory afferent inputs on the corticopontocerebellar pathway may cause contralateral hypoperfusion (Serteser et al., 2001). Remote edema after middle cerebral artery occlusion is discussed as movement of extravasated protein from the lesion (Izumi et al., 2002). Similar temporal changes with an early cell proliferation up to day 7 which was thereafter followed by a decrease have been reported for the contralateral subventricular zone after transient middle cerebral artery occlusion (Qi et al., 2007).

There is no consensus whether newly formed neurons originate from stem cells located in "non-neurogenic" cortical layers or migrate from neurogenic zones in the adult brain. The lack of clear differences in the number of cells within our ROI I and II at any evaluated time allows for the following conclusion: Either, the newly formed neurons in the cortex have no migratory background from the neurogenic zones, but are rather derived from cortical regions. Or alternatively, migration does occur from both of this so called neurogenic zones (DG and subventricular zone), but ceases early, *i.e.*, before day 9. In the present study, it is rather unlikely that newly formed neuronal cells originate from the DG because CSD alone or combined with 2-VO compared to sham operated animals did not further raise neurogenesis on days 9 and 28 in the ipsilateral DG but in the cortex. The relative low rate of proliferation and differentiation of cells in the DG seen in our study compared to a solid neurogenesis in the adult DG induced by a continuous infusion of 1μL KCl (4 M) per hour over 48 hours (Urbach et al., 2008) may be explained by the weak signaling from ten induced CSD by 10 mM KCL within a time window of 60 minutes. In addition, the continuous infusion of KCl (4 M, 1 μL/h over 48 hours) induces dividing radial glia, neural progenitor and neural precursor cells in cortical layer I up to day 3 (glial fibrillary acidic protein, vimentin and nestin immunostaining) and new immature neurons (BrdU- and beta-tubulin III; Xue et al., 2009). Although the authors could not identify whether nestin/vimentin positive cells appearing in cortical Layers V to VI are neural precursors originating from cortical layer I, they propose that the astrocytes residing in the subpial zone of the adult cortex are latent or quiescent neural progenitors which can be triggered by multiple SD waves to divide. Yanamoto et al. (2005) detected BrdU-positive and β-tubulin III double-labelled cells in "non-neurogenic regions 48 hours after SD induced by continuous KCl-infusion. These cells increased on day 6 and/or day 12 in the caudate putamen, in the frontoparietal cortex (restricted to the cortical layer V–VI) and in the anterior cingulate cortex, but not in the vehicle group. Since these ectopic new neuron-like cells did not express the marker for migrating neuroblasts (at least for 12 days), the authors suggest that these cells are not migrating or differentiating as new neurons from the subventricular zone.

Nonetheless, independent of its origin the determined fate of neuronal progenitors is controlled by signaling molecules within the niche or microenvironment of the brain (Jagasia et al., 2006). Palmer et al. (2000) investigated the proliferation and differentiation of neural stem cells in the hippocampal DG in close proximity of blood vessels, indicating possible participation of endothelial cells in the regulation of neural stem cell proliferation (Shen et al., 2004). Thus, endothelial cells foster conditions leading to local proliferation of endogenous brain cells. Even so, numerous studies suggest that the vast majority of neuroblasts die primarily by apoptotic mechanisms, and only a small population of newly generated neurons in response to ischemic insults survive long enough to mature into potentially functional neurons (Arvidsson et al., 2002; Jin et al., 2003; Zhang et al., 2004). This may explain reductions in the total number of cells after 28 days as seen in this study. Our study further supports the premise that the combination of CSD and ischemia stabilizes long-term neurogenesis in the cortex. It is clear, however, that for newly arrived cells to survive, the environment must be receptive, provide trophic support and eventually foster their integration within the tissue. Then, synaptic contacts within the surrounding parenchymal circuitry are required for differentiation and survival of newly generated cells (Gould et al., 1999a). Trophic factors like vascular endothelial growth factor, basic fibroblast growth factor and brain-derived neurotrophic factor (BDNF), in addition to being angiogenic, have strong chemotactic effects on stem-like cells and promote the survival and viability of these cells. CSD is known to increase BDNF levels in the brain (Kokaia, 1993; Yanamoto et al., 2005). The amplification of the expression of these trophic factors in neurons and glial cells by spreading depression protects against subsequent focal brain ischemia. This phenomena may contribute to neuroprotection induced by CSD (Matsushima et al., 1998). But our study points out that in combination with neuronal/cortical injury, CSD exacerbates the damage by ischemia. In this case, ischemia starts a wide range of pathways which lead to a microenvironment that favors cell death as well as neurogenesis. Important players after ischemia are microglia, endothelial cells and blood degradation products. Microglia are activated, move towards the infarct site and secret pro-inflammatory cytokines which hinder cell proliferation and neurogenesis in neurogenic regions such as subventricular zone (Solano Fonseca et al., 2016). On the other hand, secretion of anti-inflammatory cytokines such as interleukin-4 by the microglial subpopulation M2 supports proliferation and neurogenesis in a model of transient middle cerebral artery occlusion (Choi et al., 2017). According to a previous study (Kaido et al., 2012), inflammation was induced after CSD + 2-VO, so it could have influenced neurogenesis in this study. Degradation of coagulated blood releases carbon monoxide which has been shown to reduce apoptotic cell death and hence supports neurogenesis (Almeida, 2016). This might also be linked to a necessary contact of stem cells to the laminin protein α6β1 integrin. Ischemia upregulates integrins in the penumbra (Li et al., 2012) and the connection between stem cells and α6β1 integrin has been shown to promote stem cell proliferation and survival in cell culture (Rosa et al., 2016). Thus, the survival of stem cells after CSD combined with 2-VO seems to

be a multifactorial mechanism. Further studies are necessary to elucidate the origin of newly formed neurons, pathways which promote neurogenesis and distinguish between effects of 2-VO with CSD and with pharmacologically suppressed CSD.

Results from this study provide evidence for neurogenesis in the cerebral cortex after CSD and its combination with 2-VO. Our findings have shown that CSD in combination with 2-VO has an early (9 days) stimulating effect on neural stem cells in the cortex, and this effect can be maintained for at least 28 days after ischemia/CSD. This is likely attributed to the direct (2-VO) and indirect (CSD) effects of tissue ischemia. Our findings suggest that apart from the DG, the microenvironment in the cerebral cortex after CSD + 2-VO supports cell proliferation and differentiation, and also leads to long-term sustainability in the numbers of newly generated cells. We postulate that induced neurogenesis after CSD especially in the cerebral cortex is rather a direct effect of ischemia rather than CSD in itself.

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