Induction of Neurogenesis in the Adult Dentate Gyrus by Cortical Spreading Depression

Anja Urbach, PhD; Christoph Redecker, MD; Otto W. Witte, MD

Background and Purpose—Spreading depression (SD) is an epiphenomenon of neurological disorders, like stroke or traumatic brain injury. These diseases have been associated with an increased neurogenesis in the adult rodent dentate gyrus. Such proliferative activity can also be induced by conditions that—like SD—coincide with a disturbed neuronal excitability, eg, epilepsy. Thus we hypothesized that SD might likewise influence hippocampal neurogenesis and potentially act as mediator of injury-induced neurogenesis.

Methods—Repetitive cortical SD were induced by epidural application of 3 mol/L KCl. At different time points thereafter dentate gyrus neurogenesis was investigated by means of intraperitoneal bromodeoxyuridine injections and immunocytochemistry. Spatial learning and memory was tested in a Morris water maze.

Results—Cortical SD significantly increased proliferative activity in the ipsilateral subgranular zone on days 2 and 4. We detected about 280% more newborn cells in the dentate gyrus of rats that received bromodeoxyuridine during the first week after SD and were allowed to recover for 6 weeks. Most of these cells expressed the mature neuronal marker NeuN. The mitogenic action of SD was suppressed by systemic administration of the NMDA receptor antagonist MK-801. Behavioral performance of SD animals in the Morris water maze did not improve significantly.

Conclusions—From our data we postulate that the increased dentate gyrus neurogenesis observed after brain injury may at least partly be mediated by SD-like epiphenomena. Furthermore they indicate that even a strongly enhanced dentate gyrus neurogenesis may occur without significant improvements in hippocampus-dependent spatial learning and memory. (Stroke. 2008;39:3064-3072.)

Key Words: spreading depression ■ neurogenesis ■ dentate gyrus ■ cognition

Spreading depression (SD) is characterized by a slowly propagating wave of neuronal and glial depolarization accompanied by a transient suppression of neuronal activity, a failure in ion homeostasis, as well as changes in blood flow, energy metabolism, gene and protein synthesis, without causing irreversible damage to the brain.1–4 SD appears to be the underlying mechanism of the migrainous aura,5 has been associated with experimentally induced brain trauma,6 as well as ischemic stroke where it has been related to secondary tissue damage.7,8 Recently, the occurrence of SD-like events in response to brain injury could also be verified in the human brain.9

In the adult mammalian brain neurogenesis basically persists in two regions, the subventricular zone of forebrain lateral ventricles10 and the subgranular zone (SGZ) of the dentate gyrus (DG).11,12 Proliferative activity in these neurogenic zones is modulated by certain physiological and pathophysiological conditions, including behavioral activity,13–15 epilepsy,16 and ischemic injury.17,18 Many of the adult born cells die within the first weeks.19 The remaining cells differentiate mainly into neurons and become functionally integrated into the preexisting neuronal networks.20,21 Experimental data suggest that adult hippocampal neurogenesis might play a role in hippocampus-dependent learning and memory processes.13,22–24 However, to date the functional significance of adult hippocampal neurogenesis remains ambiguous.

The present study was designed to investigate whether cortical SD (CSD) influence hippocampal neurogenesis. Because SD occur in association with ischemic brain injury, which is known to affect cell proliferation in the DG, there is reason to hypothesize that SD may be at least one of the underlying mechanisms of stroke-induced hippocampal neurogenesis. First, we evaluated dentate gyrus cell proliferation and differentiation, and the rate of caspase-3–dependent apoptosis after CSD using immunocytochemical techniques. Additionally, spatial learning and memory performance of the animals was evaluated in a Morris water maze.

Materials and Methods

Animals and Surgery

The experiments were carried out on a total of 55 male Wistar rats (280 to 320 g, supplied by IVTK Jena). Animals were housed under 12-hour light/dark conditions with ad libitum access to food and...
water. For surgery animals were anesthetized with 2.5% isoflurane in an O2/N2O mixture of 1:2 and fixed in a stereotactic frame. Body temperature was maintained at 37±0.5°C. Two burr holes (AP -5, ML 3 mm and AP +2, ML 2 mm, relative to Bregma) were drilled over the left hemisphere leaving the dura intact. Direct current (DC) potentials were monitored from the anterior position. After reduction of anesthesia to 1.5% isoflurane (1:500), animals were deeply anesthetized with diethyl ether and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer. For immunohistochemistry every sixth 40-μm coronal section was rinsed in Tris-buffered saline (TBS), blocked for 30 minutes in 0.6% H2O2, and denatured in 2 N HCl for 30 minutes at 37°C. Thereafter sections were neutralized in 0.1 mol/L borate buffer at 37°C and carried out according to the guidelines on the ethical use of animals.

**BrdU Injection and Immunohistochemistry**

We used the thymidine analog bromodeoxyuridine (BrdU; Sigma-Aldrich) as a marker for dividing cells. Rats were randomly assigned to 3 groups with different BrdU injection schemes and survival times. Two groups received intraperitoneal injections of BrdU every 8 hours at day 2 (CSD, n=5; sham, n=4; MK-801, n=4) or day 4 (CSD, n=6; sham, n=4) and were allowed to recover for 1 day. Another group was injected with BrdU twice a day during the first week after surgery and euthanized at day 42 (CSD; n=9, sham: n=4).

Animals were deeply anesthetized with diethyl ether and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer. For immunohistochemistry every sixth 40-μm coronal section was rinsed in Tris-buffered saline (TBS), blocked for 30 minutes in 0.6% H2O2, and denatured in 2 N HCl for 30 minutes at 37°C. Thereafter sections were neutralized in 0.1 mol/L borate buffer at 37°C and carried out according to the guidelines on the ethical use of animals.

**Data Quantification and Statistical Analysis**

Total numbers of BrdU-positive cells were counted in every sixth section throughout the subgranular and granular cell layers of the entire DG using a Zeiss Axiosplan 2 microscope at 200× and 400× magnification. The resulting numbers were multiplied by 6 to obtain the total numbers of BrdU-positive cells of the complete DG. For phenotypization random fields of DG containing BrdU-positive cells were selected and z-stacks were scanned by confocal laser microscopy (LSM510, Zeiss). In total, the phenotypes of 50 cells per DG were determined. The percentage of colabeled cells was calculated, and absolute numbers were obtained by multiplication of the percentage with the total numbers of BrdU-positive cells.

Statistical analysis of cell numbers was performed using the Sign and Wilcoxon test for dependent and the Mann–Whitney test for independent data (all exact, 2-tailed). For behavioral data we used analysis of variance with repeated-measures (RM-ANOVA) followed by t test. Statistical significance was set at P≤0.05. Data represent mean±SEM.

**Results**

Application of 3 mol/L KCl induced approximately 7±2 CSD in a time window of 100 to 120 minutes (Figure 1A). Rats with fewer CSD were excluded from further analysis. In sham animals no DC deflections emerged (Figure 1C). Both 3 mol/L KCl and NaCl induced small cortical lesions at the application site as previously observed by others.
Cortical Spreading Depression Increases Absolute Numbers of Newborn Cells in the Adult Dentate Gyrus

CSD caused a massive induction of proliferative activity in the DG of adult rats (Table 1 and Figure 2A). BrdU immunohistochemistry, performed at day 3 after a series of 3 BrdU injections at day 2, labeled about 270% more nuclei in the ipsilateral DG relative to sham. There was a tendency for a further increase of mitotic activity at day 4. At that time, proliferation was approximately 470% higher estimated at day 5 in ipsilateral DG compared to sham. At the early time points nearly all BrdU-labeled nuclei were small, irregularly shaped, and lay in clusters in the SGZ (Figure 2A). The clusters were more numerous and larger in ipsilateral SGZ of CSD rats compared to the contralateral or sham.

For examination of the long-term fate of the mitotically active cells, BrdU was applied twice a day for 1 week and specimens were analyzed 5 weeks after the last injection. Total numbers of BrdU-positive nuclei in the ipsilateral DG of CSD rats exceeded that of sham animals by 280%. Most of the BrdU-labeled cells appeared to have migrated and dispersed from the SGZ into the inner core of the granule cell layer (Figure 2A). BrdU-positive nuclei were larger in size and more round-shaped, a morphology characteristic of surrounding mature granule cells.

In all CSD groups the total counts of BrdU-positive nuclei were significantly higher in ipsilateral compared to contralateral DG (Table 1 and Figure 2A). The contralateral DG of CSD rats also contained more BrdU-positive cells relative to sham (not significant; observed at all time points), but they were considerably fewer than in the ipsilateral DG.

At any time point, we observed similar BrdU incorporation into the DG in sham and untreated rats (Table 1). Thus, albeit the small cortical lesion caused by the application of hyperosmolar NaCl, the extent of cell birth in sham animals did not differ from basal conditions. In CSD groups higher interindividual variability in numbers of newborn cells was detected at any given time point. However, none of these animals displayed the same BrdU staining pattern as observed in controls.

We included one experimental group receiving a single injection of the noncompetitive NMDA receptor antagonist MK-801 (3 mg/kg bodyweight) before the administration of KCl to exclude the possibility that the proliferative effect observed in the DG was a direct potassium effect (potentially caused by diffusion of KCl from the cortical application site to the hippocampus) rather than a result of CSD propagation. Inhibi-
tion of the NMDA receptor has been reported to restrain the occurrence of SD.\textsuperscript{26} Proliferation was analyzed 3 days after surgery with 3 injections of BrdU at day 2 (50 mg/kg). In our experiments, CSD were completely abolished in 2 rats (Figure 1B), although one animal showed a small DC-deflection and one a single CSD at the beginning of the recording period. In all MK-801–treated animals BrdU was integrated into the ipsilateral DG to the same extent as in contralateral DG. Total numbers of BrdU-positive cells remained nearly at baseline levels found in the sham animals (Table 1; Figure 2C).

Figure 2. Number and distribution of newborn cells in the DG. A, CSD substantially increased BrdU-positive cell numbers in the ipsilateral DG. Immediately after CSD BrdU-positive nuclei were irregularly shaped and lied in clusters within the SGZ. Analyzes performed at day 42 revealed that most of the BrdU-labeled cells appear to have differentiated into mature granule neurons with large and round nuclei, which resided separately in the inner core of the granule cell layer and along the SGZ. Scale bars: 20 \textmu m. B, KCl-induced cell proliferation was almost completely abolished by systemic treatment with the NMDA-receptor antagonist MK-801; (\(P<0.05^*\) or \(P<0.01^{**}\)).

Spreading Depression Increases Neuroneogenesis and Does Not Induce Classical Apoptosis in the Adult Dentate Gyrus

As a next step we determined the phenotype of the newborn cells using triple immunofluorescence with antibodies against BrdU in combination with neuronal and glial markers. At the early time points about 80\% of the BrdU-positive cells were colabeled with the early neuronal marker doublecortin (DCX; Figure 3A and 3E; Tables 1 and 2). This observation was
Figure 3. Phenotypization of newborn cells in the ipsilateral DG of CSD rats. A, From left to right: newborn DCX-positive neuronal progenitors, S100β-positive astroglia, lectin-positive microglia, and NG2-positive synantocyte/oligodendrocyte precursor 3 days after CSD. Newborn granule neurons (BrdU+/NeuN+) and astrocyte (BrdU+/GFAP+) (B), and BrdU, DCX, and S100β triple-stained sections (C) 42 days after CSD. D, Neither in the SGZ nor in the granule cell layer activation of caspase-3 could be detected after CSD. E and F, Quantification of BrdU-positive cell populations. E, At early time points most of the newborn cells displayed features of neuronal progenitors. Whereas there was no difference in the percentage of BrdU/DCX double-positive cells between the groups, absolute numbers were significantly increased because of CSD. F, After 6 weeks BrdU/DCX colabeling completely disappeared. At this time the majority of the newborn cells had differentiated into mature neurons in both groups. SD rats showed significantly more new neurons in the ipsilateral DG and a 5% shift toward neuronal differentiation. (Scale bars: 20 μm; P<0.05* or P<0.01**).
Table 2. Absolute Numbers of Different BrdU-Positive Cell Populations (mean±SEM)

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<tr>
<th></th>
<th>Ipsilateral Mean±SEM</th>
<th>Contralateral Mean±SEM</th>
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<tr>
<td>3 Days</td>
<td></td>
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<tr>
<td>SD</td>
<td>BrdU&quot; abs. 4893.6±912.4§$</td>
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<td></td>
<td>DCX&quot; abs. 3906.5±778.6§$</td>
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<td></td>
<td>S100β&quot; abs. 81.7±33.5</td>
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<td>NeuN&quot; abs. . . . . .</td>
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<td>GFAP&quot; abs. . . . . .</td>
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<td></td>
<td>NG2&quot; abs. 391.5±183.5*</td>
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<tr>
<td>SD</td>
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<td></td>
<td>DCX&quot; abs. 1057.7±13.1</td>
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<td></td>
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<td></td>
<td>NeuN&quot; abs. . . . . .</td>
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<td>GFAP&quot; abs. . . . . .</td>
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<td></td>
<td>NG2&quot; abs. 20.7±20.7</td>
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<td>42 Days</td>
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<tr>
<td>SD</td>
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*Statistical significance relative to sham (P<0.05); §Differences to the contralateral side (P<0.05).

made bilaterally in CSD as well as in sham DG, indicating no effect of CSD on fate choice toward the neuronal lineage. Forty-two days after CSD BrdU/DCX-colabeling disappeared almost completely (Figure 3C and 3E). Instead, in both groups most of the BrdU-positive cells expressed NeuN, a feature of mature neurons (Figure 3B and 3F). Furthermore, the percentage of BrdU/NeuN double-labeled cells was higher in CSD compared to sham animals (90.0% versus 85.0%; Table 1). This indicates a facilitation of neuronal differentiation or a selective support of newborn neuron survival within the DG by CSD. With respect to the total number of BrdU-positive cells we detected a significant increase of newborn neuronal precursors after 3 and 5 days (270% and 460%, respectively) and adult neurons after 6 weeks (305%) in the ipsilateral DG after CSD (Figure 3E and 3F; Table 2). By morphological criteria the BrdU/NeuN double-labeled cells could not be distinguished from the surrounding granule cells. Taken together, CSD induced massive proliferation of precursor cells in the ipsilateral DG, newborn cells survived for at least 6 weeks and differentiated mainly into neurons.

Only a small subset of the newborn cells showed astrocytic or microglial characteristics. These cell types differed neither in their percentage nor in absolute numbers between groups (Tables 1 and 2; Figure 3A and 3B). However, the third glial cell type analyzed, the NG2-positive synanchocytes (Figure 3A), were significantly increased concerning absolute numbers in the ipsilateral DG 3 days after CSD compared to controls (Table 2). Absolute numbers at later time points as well as the percentage of BrdU/NG2 double-positive cells did not significantly differ from controls.

We further investigated the rate of apoptosis in the DG after CSD. Coronal sections were double-labeled with antibodies against BrdU and cleaved caspase-3. We never detected active caspase-3 staining in the DG of CSD or sham animals (Figure 3D). Only the salt lesions exhibited cells with active caspase-3 immunoreactivity (data not shown).

DC Recordings in the Hippocampus During Cortical Spreading Depression

To ensure that the application of KCl to the cortex did not induce SD in the hippocampus we performed recordings of the hippocampal DC potential in a group of 4 rats which underwent CSD. In these animals, no hippocampal DC deflections were observed in a time course of 3 hours after the first KCl application (which persisted for 2 hours), indicating that SD did not occur in the hippocampus (Figure 1A).

Cognitive Performance in the Morris Water Maze Task

To assess whether CSD were associated with alterations in hippocampal function we tested rats in a spatial version of the MWM. During the training sessions time to reach the hidden platform did not differ between the groups (Figure 4B). Escape latency decreased equally for both groups across the sessions. The overall mean latency was 19.4±1.3 s in CSD animals compared to 23.7±3.1 s in the sham group. Furthermore, we analyzed the percentage of time spent in the target quadrant during acquisition of MWM task. Group values did not differ from session 1 to 6. However, CSD animals spent more time in the target quadrant during the last session at day 7 (Figure 4C).
Rats underwent probe trials without a platform on day 7, 22, and 43 after the beginning of the MWM to test for a potential influence of CSD on memory retention (Figure 4D). There was no difference between CSD and sham animals at either time point. On probe trial 1 both groups showed a 55% preference for the target quadrant. Two weeks later, the percentage of time spent in the target quadrant declined to 22.2 ± 3.5% in CSD and 24.0 ± 4.5% in sham animals. Similar results were obtained in the last probe trial. As rats tended to abandon searching in the former target quadrant when recognizing the absence of the platform we additionally analyzed the probe trials in 15-s intervals. Likewise, no differences between the groups could be detected (data not shown). Swim speed and thigmotactic behavior during training and probe trials did not differ between the groups.

**Discussion**

The present study was designed to evaluate the consequences of CSD on neurogenesis in the adult hippocampus. Furthermore, we wanted to determine whether CSD-mediated changes could bias hippocampus-dependent learning and memory. We had several reasons to assume that CSD could modify hippocampal neurogenesis: first, SD is a known epiphenomenon of neurological disorders like trauma or focal ischemic injury which are linked to an increased neurogenesis in this region. Second, other conditions that, like SD,
coincide with a perturbed neuronal excitability (like epilepsy or electroconvulsive shock) also exhibit neurogenesis-inducing potential.\textsuperscript{16,28} Furthermore, as demonstrated by a recent study, SD has the capability to activate neurogenesis in the subventricular zone of the lateral ventricle, the second structure in the brain that generates new neurons throughout life.\textsuperscript{30} However, no data exist on the impact of CSD on neurogenesis in the hippocampal formation.

Here, we showed that repetitive KCl-induced CSD dramatically increased ipsilateral cell proliferation in the adult rat SGZ. The majority of cells that were born during the first week and analyzed 42 days after CSD adopted the phenotype of mature granule neurons that were primarily distributed within the inner core of the granule cell layer. This resulted in an increment of newborn neurons in the ipsilateral DG of CSD animals of about 305%. This effect was based not only on the increase in absolute numbers of newborn cells but also on the relative amount of new neurons which was enhanced by 5% relative to sham. Because the percentage of newborn neuronal progenitors at the early time points was comparable in CSD and control rats we conclude that, beside the facilitation of proliferation of precursor cells, CSD promote the survival of new neurons rather than changing the fate choice of precursor cells. Pretreatment with the NMDA receptor antagonist MK-801 before KCl application abolished CSD and the increase in BrdU-positive cell numbers within the ipsilateral DG. In addition, recordings of the hippocampal DC-potential during CSD revealed that SD did not invade into this structure. These control experiments proved that CSD rather than diffusing KCl or SD spreading to the hippocampus account for the observed mitogenic effects within the DG.

How CSD facilitates neurogenesis in the DG remains a matter of speculation. First to note, the DG receives its major excitatory input via the perforant path originating from layer II/III neurons of the entorhinal cortex.\textsuperscript{30} Furthermore, SD are accompanied by an increased release of glutamate.\textsuperscript{31} Passing the entorhinal cortex CSD might activate the perforant path inducing the liberation of glutamate from cortical afferents to the DG which in turn might excite the hippocampal network and modulate neurogenesis in this region. In support of this hypothesis, it was demonstrated that CSD significantly enhance synaptic long term potentiation in CA\textsubscript{1}\textsuperscript{32} and drive c-FOS protein expression in the ipsilateral granule cell layer,\textsuperscript{32} and that tetanic stimulation of the perforant path increases dentate progenitor proliferation in an NMDA receptor dependent manner.\textsuperscript{33} An involvement of glutamatergic excitatory input in adult neurogenesis has been suggested from different experimental data, even though it seems to have opposing effects under physiological and pathophysiological conditions. In the native brain, NMDA receptor agonists decreased and antagonists increased neurogenesis.\textsuperscript{34,35} In contrast, NMDA receptor blockade suppressed neurogenesis induced by focal ischemia\textsuperscript{36} or tetanic stimulation of the perforant path.\textsuperscript{33}

Several studies suggested a close correlation between the number of new granule neurons and the cognitive performance of animals in the MWM.\textsuperscript{23,24,37} Considering the remarkable increase in the number of new cells in the present study, the observed barely significant improvement in functional performance does not support such a direct correlation. The lack of correlation between performance in the MWM and the number of new neurons in the hippocampus was also reported in other studies.\textsuperscript{22,38,39} These authors furthermore showed that spatial learning in the MWM remains also possible in the absence of newly generated cells, whereas associative learning in trace conditioning paradigms was impaired.\textsuperscript{22,29,40} Recent studies suggested that the way in which new dentate granule neurons mature and integrate into the existing circuitry is markedly affected by the conditions under which these cells evolve.\textsuperscript{41-42} Jakubs and coworkers demonstrated that cells born in an epileptic environment differed significantly from those born under physiological conditions concerning synaptic drive and short-term plasticity of their afferent connectivity.\textsuperscript{41} One might therefore speculate that it is not only the number of new neurons in the granule cell layer, but their precise functionally correct integration into the hippocampal network which determines their impact on hippocampal memory processes.

Our study provides evidence that small cortical lesions per se do not influence hippocampal neurogenesis. Neurogenesis was exclusively induced in rats that underwent CSD. The DG of sham operated control rats contained similar numbers of BrdU-positive cells as untreated native animals. To remind—in sham animals epidural application of 3 mol/L NaCl induced small cortical lesions without appearance of SD. In support of this finding Arvidsson and coworkers\textsuperscript{36} observed that proliferative activity in the DG after middle cerebral artery occlusion was independent of the degree of cortical damage. Further investigation is required to evaluate whether larger lesions to the cortex without CSD lack neurogenesis inducing potential as well.

Moreover, our data allowed us to speculate that SD-like events serve as one of the underlying mechanisms of stroke or traumatic brain injury induced neurogenesis. Both of these diseases augment cell proliferation in the DG that resembles the extent and pattern observed after CSD in our study.\textsuperscript{17,27,36,43} Both are accompanied by SD-like phenomena, eg, the so-called peri-infarct depolarizations, which can be blocked by NMDA receptor antagonists.\textsuperscript{44} Furthermore, these drugs have been shown to prevent ischemia-induced neurogenesis.\textsuperscript{36,45} Based on our results we hypothesize that SD-like epiphenomena rather than the lesion itself might mediate injury-induced neurogenesis. NMDA receptor antagonists might prevent stroke-induced neurogenesis in the DG not directly but rather indirectly by blocking concomitant peri-infarct depolarizations. These speculations would also explain the apparently conflicting data on the action of NMDA receptor antagonists in basal and pathologically induced DG neurogenesis.\textsuperscript{34-36,45}

In summary we have shown that CSD has the potential to induce neurogenesis in the adult dentate gyrus. Because several recent studies demonstrated SD-like phenomena in association to migraine, human brain trauma, and subarachnoidal hemorrhages,\textsuperscript{9,46,47} experimental studies of CSD are of great significance for certain human brain pathologies. To what extent CSD also occur in ordinary stroke patients needs to be investigated in further clinical studies.

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Disclosures
None.

References
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