Constraint-Induced Movement Therapy Enhanced Neurogenesis and Behavioral Recovery after Stroke in Adult Rats

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Constraint-induced movement therapy (CIMT) has been extensively used for stroke rehabilitation. CIMT encourages use of the impaired limb along with restraint of the ipsilesional limb in daily life, and may promote behavioral recovery and induce structural changes in brain after stroke. The aim of this study was to investigate whether CIMT enhances neurogenesis in rat brain after stroke that was generated by middle cerebral artery occlusion. Adult rats were divided into sham group, ischemia group and ischemia treated with CIMT group. Rats of CIMT group were treated with a plaster cast to restrain the healthy forelimb for 14 days beginning 1 week after ischemia. The proliferation of neuronal cells labeled with bromodeoxyuridine (BrdU) and behavioral recovery were analyzed at day 29 after ischemia. We also measured the tissue level of stromal cell-derived factor 1 (SDF-1) by ELISA. SDF-1 might be involved in the regulation of neurogenesis following stroke. In the subventricular zone of the animals treated with CIMT, there was a significant increase in the number of BrdU-positive cells (135 ± 18 , $P < 0.05$), compared with ischemia group (87 ± 12) or sham group (18 ± 3.6). Likewise, in the dentate gyrus, animals treated with CIMT showed a significant increase in BrdU-positive cells (296 ± 26 , $P < 0.05$) compared with ischemia group (225 ± 18) or sham group (162 ± 11). CIMT treatment after stroke significantly improved behavioral performances and increased the SDF-1 protein levels in the cortex and dentate gyrus. In conclusion, CIMT treatment enhances neurogenesis and functional recovery after stroke. ——— Constraint-induced movement therapy; neurogenesis; cerebral ischemia; stromal cell-derived factor 1; functional recovery.

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Constraint-induced movement therapy (CIMT) has been extensively used for stroke rehabilitation (Blanton et al. 2008; Cramer and Riley 2008). This approach, which encourages use of the impaired limb along with restraint of the ipsilesional limb in daily life, has provided substantial evidence of efficacy for promoting behavioral recovery and enhancing functional reorganization of brain. There were many studies using neuroimaging techniques to assess functional alterations of CIMT in stroke patients (Hamzei et al. 2006; Sawaki et al. 2008; Tarkka et al. 2008). A recent human study using voxel-based morphometry, a quantified structural MRI technique, showed significant increases in gray matters of cortex and hippocampus induced by CIMT, which were accompanied by large improvements in behavioral outcome (Gauthier et al. 2008). However, the histological basis for these structural changes remains unclear, but it may involve dendritic arborization, synaptogenesis, or neurogenesis. A recent animal study showed that forced use of the impaired limb enhanced the growth and synapse formation of the corticospinal tract (CST) fibers after unilateral CST injury. Moreover, morphological changes of CST fibers were correlated with significant behavioral recovery evaluated by horizontal ladder test in rats with restriction of

unimpaired forelimb (Maier et al. 2008).

Neural stem cells which are located at subventricular zone (SVZ) and hippocampal subgranular zone in the adult mammalian brain continuously produce newborn neurons that can functionally integrate by forming synapses with the existing neural circuitry (Danzer 2008; Eisch et al. 2008). Accumulating evidence indicated that adult neurogenesis dramatically responded after brain injury (Zhang et al. 2008; Kuge et al. 2009). More importantly, ablation or blockade of neurogenesis in these regions after stroke worsened outcomes of stroke, suggesting that brain injury induced neurogenesis (Raber et al. 2004). Therefore, we speculated that the increases in gray matters observed in the hippocampal region or cortex of the brain after CIMT treatment might be partly due to the enhanced neurogenesis process. As direct measurement of neurogenesis is impossible in human, animal studies are needed to investigate the effects of CIMT on neurogenesis after experimental stroke and valuable for uncovering principals of structural plasticity changes induced by CIMT.

Stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 were found to play an important role in both the developing brain and adult brain (Lu et al. 2002; Peng et al.

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2007; Stumm and Holtt 2007; Bhattacharyya et al. 2008). SDF-1 and CXCR4 were highly expressed throughout the SVZ and subgranular zone of the dentate gyrus and involved in the migration, proliferation, differentiation of neural progenitor/stem cell (Lu et al. 2002; Stumm et al. 2002; Tissir et al. 2004; Bhattacharyya et al. 2008). Upregulation of SDF-1 immunoreactivity was detected in the ischemic penumbra. Moreover, SDF-1/CXCR4 signaling directed the migration of new neurons generated from endogenous neural stem cells toward sites of ischemic damage and mediated homing of transplanted neural stem cells to the infarct area after stroke (Stumm et al. 2002; Hill et al. 2004; Robin et al. 2006; Thored et al. 2006).

This study investigated the effects of CIMT on neurogenesis in subventricular zone and hippocampal subgranular zone and behavioral recovery after stroke, as well as the expression of stromal cell-derived factor 1 (SDF-1). Thus, SDF-1 may be involved in the regulation of neurogenesis following stroke.

Materials and Methods

Experimental Groups

All experiments were conducted in accordance with the Guidelines for Animal Experimentation at the Institutional Animal Care and Use Committee of China Medical University. Male Wistar rats (200–250 g) were randomly assigned to three groups: sham ($n = 5$), ischemia ($n = 8$) and ischemia treated with CIMT ($n = 8$). Rats of CIMT group were treated with a plaster cast of the ipsilateral side for 14 days beginning 1 week after ischemia. All the animals were kept under standard housing conditions in ordinary cages (5 animals per cage).

Middle cerebral artery (MCA) ischemic-reperfusion model

We induced transient focal cerebral ischemia which was allowed to reperfuse after 90 min by intraluminal middle cerebral artery occlusion (MCAO) with a monofilament according to Longa et al. (1989). Briefly, rats were anaesthetized with 10% Chloral Hydrate (400 mg/kg, i.p.). The cervical vessels were exposed through a median ventral cervical incision in the skin and the left common artery (CCA), the left external carotid artery (ECA) and the left internal carotid artery (ICA) were isolated. The ECA and CCA were ligated close to its origin. A 3-0 silk suture was tied loosely around the ICA, and a microvascular clip was placed distally across the ICA. A puncture was performed at the ICA-ECA bifurcation and a 4-0 polyester monofilament thread with its tip rounded by heating, was introduced into the ICA. The suture around the ICA (and the polyester thread) was tightened, and the microvasculature clip was removed. The polyester thread was

then gently advanced into the ICA lumen on a length of 18 ± 0.5 mm until it reaches and occludes the ostium of the left MCA. 90 min after occlusion, the filament was withdrawn to allow reperfusion. The sham-operated rats underwent same surgical procedure and the polyester thread was advanced into the ICA on a length of 9 mm. The rectal temperature was maintained at 37.0 – 37.5°C with a heating pad throughout the surgical procedures.

Constraint-induced movement therapy

CIMT-treated animals were fitted with a plaster cast as described previously (Muller et al. 2008). The upper torso was wrapped in soft felt, and the ipsilateral forelimb was wrapped in felt and positioned in a naturally retracted position against the animal's sternum. Plaster of Paris strips were wrapped around the immobilized limb and upper torso (Muller et al. 2008). CIMT-treated animals were forced to rely on their impaired completely in standard home cage without daily training.

BrdU labeling

BrdU (Sigma, St. Louis, MO, U.S.A.) was administered intraperitoneally at a dose of 50 mg/kg twice daily at 8 hrs intervals for three consecutive days, 24 hrs after ischemia induction (Fig. 1).

At day 29 after MCAO, rats were anaesthetized with 10% chloral hydrate and perfused transcardially with 4% paraformaldehyde in PBS. The brains were removed and fixed for 4–6 hrs and then stored in 30% sucrose solution (prepared with 0.1 M PBS, PH 7.2–7.4) at 4°C overnight. Coronal consecutive sections ($50 \mu\text{m}$) were cut through the brain on a cryostat and stored at -20°C in cryoprotecting buffer (30% ethylene glycol, 20% glycerin, and 0.05 M PBS).

Immunohistochemistry and cell counting

BrdU immunostaining was followed as described previously (Jessberger et al. 2007). Free-floating sections were washed in potassium PBS (KPBS) 3 times for 5 min and immersed in 1% H_2O_2 at room temperature for 30 min to block endogenous peroxidase. The sections were incubated in 2 N HCl at 37°C for 45 min then washed in 0.1 M boric acid (PH 8.5) for 10 min. Sections were incubated in KPBS, 0.4% Triton X-100, and 5% normal goat serum for 30 min, followed by overnight incubation with primary anti-BrdU antibody (monoclonal rat, 1 : 200, Sigma, St. Louis, MO, U.S.A.). After rinsing, sections were incubated with biotinylated secondary antibody (1 : 200, Vector Laboratories, Burlingame, CA, U.S.A.) for 45 min, and then incubated for 1 hr in avidin-biotin-peroxidase complex (1 : 1000, Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, U.S.A.). After thorough rinses, reaction product was detected using 0.05% 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St. Louis, MO, U.S.A.) for 10 min.

Sections were viewed on an Olympus microscope with Nikon

Fig. 1. Study design. The arrows indicate the timing of operation, BrdU labeling, behavioural testing and sacrifice. The bold black line indicates CIMT treatment on day 8 to day 21 after cerebral ischemia.

digital camera, and the images were visualized on a computer monitor. For BrdU-staining, only the cells with BrdU clearly localized and confined to the nucleus were considered as BrdU-reactive cells. All of the BrdU-positive cells in the lateral ventricle wall and dentate gyrus ipsilateral to the injury were counted on every sixth sections for a total of 8 sections from each rat. Results were presented as the average number of BrdU-positive cells per section.

Infarct Volume Measurement

Rats were sacrificed by decapitation and brains were rapidly removed from the skulls and frozen in refrigerator at -20°C for 15 min until it became hard. Consecutive coronal slices (2 mm thickness) were cut using a brain slicer. The slices were immersed in 2% TTC phosphate buffer (0.2 mmol/L, pH 8.7) 10 ml at 37°C for 15 min incubation in the dark. The undamaged area was stained with red color and the ischemic area remained grey. Tissue sections were immersed in a 1.5% paraformaldehyde phosphate buffer (0.2 mmol/L, PH 7.4) at 4°C for 24 hrs. The infarct areas were assessed with a computerized image analyzer ImageJ (NIH), and the infarct volume was calculated.

Measurement of SDF-1 level

Rats were sacrificed by decapitation and brains were dissected out at day 29 after ischemia induction. Cortex tissues from surrounding area of the ischemic lesion and tissues from dentate gyrus ipsilateral to the ischemia were weighed and the total protein was extracted by a commercial protein extraction reagent (Pierce, Rockford, IL, U.S.A.) at a ratio of 1 g of tissue to 10 ml reagent according to the product manual. The supernatants were used for SDF-1 measurement. SDF-1 levels were measured using SDF-1 ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.) as described before (Dar et al. 2005). 100 μL diluted coated antibody (1 : 1000) was added into each well and was stored in moist box at 4°C for 48 hrs. Wells were washed 3 times for 5 min and 100 μL blocking liquid was added at 37°C for 1 hr. After washing wells 3 times, 100 μL diluted standard, sample, positive and negative controls were added in duplicate wells at 37°C for 1 hr. Wells were washed for 3 times and added 100 μL diluted antibody (1 : 100) for measuring at 4°C overnight then stored at room temperature for 1 hr. After rinses, 100 μL diluted enzyme labeled antibody (1 : 700) was added at 37°C for 1 hr, rinsed 3 times and 100 μL prepared ABTS developer was added at 37°C for 15-30 min. Microplate Reader at 540 nm was applied to measure optical density and generate standard curve.

Tapered/Ledged beam-walking test

The issue of the analysis of recovery of original function versus learned motor compensation is a major concern in brain injury models. A recent advance in resolving this issue is the development of a test that reduces learned compensation that otherwise masks functional capacity. In this study, the tapered/ledged beam was used to test sensorimotor function of hindlimbs. The novel feature of the task is that there are ledges along both sides of the beam, which are 2 cm lower than the upper surface of the beam. This allows assessment of functional outcome relatively independent of practice effects and learned compensation because the ledges provide a crutch on which the animal can place the forelimbs or hindlimbs that slip off the upper beam (Schallert et al. 2002; Schallert and Woodlee 2005). Compensatory adjustment in posture or weight bearing in nonimpaired limbs becomes unnecessary when the animal traverses the

beam. Another feature is that the beam is wide at the starting point and tapers gradually to a narrow end near the goal, which makes the task more difficult and sensitive. The rats were pretrained for three days to traverse the beam before test. The animals were tested on postoperative days 25 (Fig. 1). The end of the beam was connected to a black box ($20.5 \times 25 \times 25$ cm) with a platform at the starting point. A bright light was placed above the start point to motivate the rats to traverse the beam. The rats' performance was videotaped and later analyzed by calculating the slip ratio of the impaired (contralateral to lesion) hindlimb (number of slips/number of total steps) (Schallert et al. 2002). Steps onto the ledge were scored as a full slip and a half slip was given if the limb touched the side of the beam. The mean of three trials was used for statistical analyses. All behavioral analyses were performed by a same observer, blind to the experimental groups.

Water-maze test

Spatial learning was analyzed with a match-to-place version of the Morris water-maze (Troy Harker and Whishaw 2002) on postoperative days 26-28 (Fig. 1). The water-maze apparatus consisted of a circular, black fiberglass pool (150 cm in diameter, 74 cm deep, filled with water at 20°C to a height of 54 cm). The top surface of the platform (10×10 cm, composed of black rubber) was 2 cm below the water line. The starting locations were called north, south, east and west, and were located arbitrarily at equal distances from each other on the pool rim. The swim paths were monitored by a video camera connected to a computer through an image analyzer (HVS image). If the rat failed to find the hidden platform within 70 s, it was placed on it. The rat was allowed to remain on the platform for 10 s. The inter-trial interval was 30-60 s. The rats were given four trials each day. Trials 1 and 3 began from one of the points located farthest from the platform and the start point was changed after each trial. The location of the platform was changed to a different quadrant each day. The escape latency (time to reach the platform) was used to assess acquisition of the water-maze task.

Statistics

Statistical differences in the number of BrdU positive cells, infarct volumes, Beam-walking data and SDF-1 levels between groups were analyzed using one-way ANOVA with Duncan's post hoc test. The water-maze data (escape latency) were analyzed using ANOVA for repeated measures.

Results

Infarct Volumes

There was no significant difference in infarct volumes between ischemia group (129.30 ± 14.64 mm³) and CIMT group (141.1 ± 10.92 mm³).

Beam-walking test

There was a significant overall group effect in slip ratio with the impaired hindlimb ($P = 0.007$) (Table 1). That is, ischemic rats were more severely impaired than sham-operated animals. There was a significant difference in slip ratio with the impaired hindlimb ($P = 0.035$) between the ischemic controls and ischemic rats treated with CIMT, suggesting that CIMT treatment significantly improved beam-walking performance.

Table 1. Slip ratio of hindlimb in the beam-walking test.

Group	Slip ratio (%)
SHAM	15.4 ± 3.3
Ischemia	46.3 ± 5.8*
Ischemia treated with CIMT	30.2 ± 4.4* [#]

Data are expressed as slips ratio (made slips/steps) for the impaired (contralateral to lesion) hindlimb. *: different from SHAM group ($P < 0.05$), [#]: different from ischemia group ($P < 0.05$).

Fig. 2. Water maze performances of rats after cerebral ischemia.

Compared with ischemia group, ischemic rats treated with CIMT had shorter escape latency. Data are expressed as mean ± S.E.M. *: different from SHAM group ($P < 0.05$), [#]: different from ischemia group ($P < 0.05$).

Water-maze test

When the water-maze data were analyzed, there was significant group effect in escape latency ($F[2,18] = 4.217$, $P = 0.035$) (Fig. 2). Compared with ischemic control, ischemic rats treated with CIMT had shorter escape latency ($P = 0.028$).

Level of SDF-1 protein

In the cortex, ischemia treated with CIMT group showed a significant increase in SDF-1 protein level (52 ± 7.1 pg/mg, $P < 0.05$) compared with ischemia group (34 ± 4.2 pg/mg) or sham group (18.5 ± 2.2 pg/mg). In the dentate gyrus, ischemia treated with CIMT group also showed a significant increase in SDF-1 protein level (172 ± 15.8 pg/mg, $P < 0.05$) compared with ischemia group (99 ± 10.5 pg/mg) or sham group (64 ± 7.8 pg/mg) (Fig. 3).

Cell counts

In the subventricular zone, ischemia treated with CIMT group showed a significant increase in BrdU positive cells (135 ± 18 , $P < 0.05$) compared with ischemia group (87 ± 12) or sham group (18 ± 3.6). In the dentate gyrus, ischemia treated with CIMT group also showed a significant increase in BrdU positive cells (296 ± 26 , $P < 0.05$) compared with ischemia group (225 ± 18) or sham group ($162 \pm$

Fig. 3. SDF-1 levels in SVZ and dentate gyrus after cerebral ischemia.

Compared with SHAM and ischemia group, CIMT treatment significantly increased the levels of the SDF-1 protein both in SVZ and dentate gyrus ipsilateral to the ischemia. Data are expressed as mean ± S.E.M. *: different from SHAM group ($P < 0.05$), [#]: different from ischemia group ($P < 0.05$).

11) (Fig. 4).

Discussion

The present study clearly demonstrated that CIMT following stroke significantly increased the neurogenesis in both subventricular zone and dentate gyrus. Behavioral assessments demonstrated that increased numbers of BrdU positive cells in the different experimental groups were correlated with improved beam-walking and spatial learning performances. We further found that 2-week treatment of CIMT after stroke significantly increased the SDF-1 protein level in cortex and dentate gyrus ipsilateral to the ischemia.

CIMT is based on the theory of “learned non-use.” Learned non-use develops during the early stages following a stroke as the patient (or lesioned animal) that has difficulty in using the affected side will quickly learn to compensate by using the unaffected side (Taub and Uswatte 2003). This compensatory strategy has been shown to hinder the intrinsic recovery of function in the impaired limb. CIMT treatment discourages the use of the unaffected limbs and encourages active use of the impaired limbs.

Many studies using neuroimaging techniques such as functional magnetic resonance image (fMRI), positron emission tomography, or transcranial magnetic stimulation demonstrated that CIMT enlarged the areas of cortical representation in affected hemisphere and activated the lesioned cortical and the brain adjacent to it, thus induced the functional recovery of the impaired limb in stroke patients (Hamzei et al. 2006; Sawaki et al. 2008; Tarkka et al. 2008). These findings suggested that cortical reorganization might play important role in the mechanism of CIMT in enhancing functional recovery (Hamzei et al. 2006; Sawaki et al. 2008). However, functional alterations in brain assessed by such neuroimaging techniques might change rapidly over time. Recently, Gauthier et al. using a quantified structural MRI technique provided new evidences

A

B

C

Fig. 4. Proliferation of newborn cells in SVZ and dentate gyrus after cerebral ischemia.

Immunohistochemistry showed CIMT treatment group had significant more BrdU positive cells indicated by arrows in both SVZ (A) and dentate gyrus (B) ipsilateral to the ischemia compared with ischemia group or SHAM group. C showed quantitative data of numbers of BrdU positive cells in SVZ and dentate gyrus. Data are expressed as mean $\pm$ S.E.M. *: different from SHAM group ($P < 0.05$), #: different from ischemia group ($P < 0.05$).

that CIMT could result in structural reorganization in the brains of stroke patients and that the magnitude of this structural change was directly correlated with significant behavioral recovery. Unfortunately, this quantified structural MRI technique did not have sufficient resolution to identify the histological basis for these structural changes. In the present study, we found that CIMT in rats with experimental stroke significantly increased the BrdU positive cells in both subventricular zone and dentate gyrus. Translating the present results from animal to human suggested that functional alterations in brain induced by CIMT might be partly supported by the increased production of newborn neurons that might migrate to an infarct area and participate in its repair process.

Neural stem cells with the potentiality of self renewal and multiple differentiations exist in central nerve system of adult animals including human (Danzon 2008; Eisch et al. 2008; Zhao et al. 2008). Under normal conditions they are quiescent and will be activated under certain signal stimulation. Accumulating evidence indicated after ischemic injury neural stem cells in hippocampus, subventricular zone and cortex could proliferate, migrate and differentiate into neurons and neuroglia cells, which could take place the function of injured neurons (Zhang et al. 2008; Kuge et al. 2009). In the present study, increased numbers of BrdU positive cells in subventricular zone and dentate gyrus induced by CIMT after stroke were correlated with improved beam-walking and spatial learning performance. The result suggested manipulation of neurogenesis process with CIMT might lead to improved rehabilitation after stroke.

A number of studies in animals and human validated that CIMT significantly improved motor function after brain injury (DeBow et al. 2003; Maclellan et al. 2005; Gauthier et al. 2009; Lim et al. 2009; Massie et al. 2009). The result of our research was in accordance with other studies. However, whether CIMT treatment after stroke improves cognitive function is largely unknown. A recent study showed that after cortical infarcts in the forelimb sensorimotor cortex, daily reaching training of the impaired paw significantly improved functional performance in the Morris water maze. The better spatial learning performance of the CIMT treatment group as demonstrated by the water maze data in the present study was consistent with this report (Wurm et al. 2007).

The mechanisms of CIMT enhancing neurogenesis in subventricular zone and dentate gyrus were poorly understood. The present results indicated that it might be correlated with the upregulation of SDF-1 levels in brain.

SDF-1 and its receptor CXCR4 is highly expressed throughout the SVZ and subgranular zone of the dentate gyrus (Lu et al. 2002; Stumm et al. 2002; Tissir et al. 2004), which are two main persistent neurogenic regions in adult brains. In addition, a recent study showed that neural progenitors expressed SDF-1/CXCR4 in adult brain (Tran et al. 2007). One major function of SDF-1/CXCR4 signaling was

to act as a chemotactic cue for the migration of neural stem/progenitor cells. SDF-1 also regulated the proliferation of these cells. Using hippocampal cultures, Kolodziej et al. (2008) showed that SDF-1/CXCR4 signaling was neurotrophic and prompted the survival of newborn neurons through Erk and Akt pathway. Moreover, SDF-1/CXCR4 facilitated the excitability of immature neurons and regulates GABAergic inputs to neural progenitors in the dentate gyrus (Kolodziej et al. 2008).

Upregulation of SDF-1 level had been detected in the ischemic penumbra coincidently with numerous CXCR4-expressing cells by immunohistochemical and ELISA method (Stumm et al. 2002; Hill et al. 2004), which was in accordance with our result. Moreover, SDF-1 had been reported to promote neuronal progenitor motility in the ischemic brain (Robin et al. 2006) and to direct the migration of new neurons toward sites of ischemic damage (Thored et al. 2006). However, the specific CXCR4 blocker AMD3100 can inhibit the migration of the newborn neurons toward the ischemic damage (Thored et al. 2006). Thus, the SDF-1/CXCR4 system appeared to play an important role in neuronal plasticity after injury, neurogenesis and repair. Upregulation of SDF-1 level by CIMT treatment after stroke may augment the self-repair ability of the adult brain, therefore improve behavioral functions.

One of the main limitations in the present study was the use of BrdU for studying neurogenesis. BrdU is a marker for DNA synthesis instead of a marker for cell proliferation and neurogenesis. Retroviruses encoding transgenes such as fluorescent proteins may be useful for the study of endogenous neurogenesis by avoiding the potential pitfalls of misidentifying BrdU incorporation in newly synthesized DNA in cells undergoing ischemia-induced apoptosis (Kuan et al. 2004), as well as gliogenesis (van Praag et al. 2002). In addition, several studies showed although substantial newborn neurons migrated into ischemic areas in the first weeks after stroke, a relatively small number of newly born neurons matured and survived in the long-term (Zhang et al. 2001; Arvidsson et al. 2002; Ohab et al. 2006). However, we did not know whether or how long these new neurons induced by CIMT after stroke could survive in the present study. Moreover, whether or how these new neurons integrated into the existing circuitry and contributed to the structure and function of the brain were unexplored.

Despite the need to elucidate these issues, this study provided the new evidence that CIMT treatment enhanced both functional recovery and structural neuroplasticity after stroke. It is meaningful for further understanding the self-repair ability of central nervous system and establishing the theory basis for clinical rehabilitation of stroke.

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