Controlled Cortical Impact Results in an Extensive Loss of Dendritic Spines that Is Not Mediated by Injury-Induced Amyloid-Beta Accumulation

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Abstract

The clinical manifestations that occur after traumatic brain injury (TBI) include a wide range of cognitive, emotional, and behavioral deficits. The loss of excitatory synapses could potentially explain why such diverse symptoms occur after TBI, and a recent preclinical study has demonstrated a loss of dendritic spines, the postsynaptic site of the excitatory synapse, after fluid percussion injury. The objective of this study was to determine if controlled cortical impact (CCI) also resulted in dendritic spine retraction and to probe the underlying mechanisms of this spine loss.

We used a unilateral CCI and visualized neurons and dendritic spines at 24 h post-injury using Golgi stain. We found that TBI caused a 32% reduction of dendritic spines in layer II/III of the ipsilateral cortex and a 20% reduction in the dendritic spines of the ipsilateral dentate gyrus. Spine loss was not restricted to the ipsilateral hemisphere, however, with similar reductions in spine numbers recorded in the contralateral cortex (25% reduction) and hippocampus (23% reduction).

Amyloid- β (A β), a neurotoxic peptide commonly associated with Alzheimer disease, accumulates rapidly after TBI and is also known to cause synaptic loss. To determine if A β contributes to spine loss after brain injury, we administered a γ -secretase inhibitor LY450139 after TBI. We found that while LY450139 administration could attenuate the TBI-induced increase in A β , it had no effect on dendritic spine loss after TBI. We conclude that the acute, global loss of dendritic spines after TBI is independent of γ -secretase activity or TBI-induced A β accumulation.

Key words: amyloid beta; brain trauma; controlled cortical impact; dendritic spine; gamma-secretase inhibitor

Introduction

THE CLINICAL MANIFESTATIONS that occur after traumatic brain injury (TBI) can include a wide range of cognitive, emotional, and behavioral deficits. While deficits in cognitive ability increase as a function of injury severity,^{1–4} episodes of depression and anxiety are as prevalent after mild TBI as they are after moderate/severe TBI.^{1–4} Temporary amnesia, headaches, and sleep disorders are also common, even after a mild TBI. The etiology of such diverse symptomology is unknown; however, the loss of excitatory synapses may explain the appearance of some of these symptoms.

Dendritic spines are tiny protrusions along a neuron's dendrite that receive excitatory input from a single synapse of an axon. Changes in dendritic spine numbers and morphology have been implicated in a diverse set of neurological disorders including Alzheimer disease, fragile X syndrome, and schizophrenia.^{5–7} Recently, a preclinical study has demonstrated that a lateral fluid percussion injury can cause

a transient reduction in the number of dendritic spines in the rat cortex.⁸ TBI-induced spine loss can be prevented with the calcineurin inhibitor FK506,⁸ demonstrating the involvement of this calcium-dependent pathway in the spine retraction process after injury. Activation of calcineurin is extremely important for rapid dendritic spine remodeling, and this mechanism has been implicated in dendritic spine loss in other neurodegenerative disorders including epilepsy, Parkinson disease, and Alzheimer disease.^{9–11}

A soluble factor known to induce dendritic spine retraction is the Alzheimer disease-related peptide, amyloid-beta (A β). The rapid accumulation of A β after TBI is well documented in both humans and experimental animal models.^{12–17} Excess A β is detrimental to dendritic spine health both *in vivo*¹⁸ and *in vitro*¹⁹ and, similar to spine loss after TBI, A β -induced dendritic spine loss can be prevented with the calcineurin inhibitor FK506.²⁰ These data suggest that spine retraction after A β and TBI may have a common mechanism of action through calcineurin activation.

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As A β accumulation and calcineurin-dependent dendritic spine loss both occur after TBI, we wanted to assess if TBI-induced A β accumulation was responsible for dendritic spine loss after brain injury. To achieve these goals, we determined the effect of controlled cortical impact (CCI) on dendritic spine levels in the cortex and hippocampus of injured mice. We also measured the impact of the γ -secretase inhibitor LY450139 on A β accumulation after TBI and its effect on injury-induced spine loss.

Methods

CCI injury

All procedures were performed in accordance with protocols approved by the Georgetown University Animal Care and Use Committee, as described previously.²¹ Three-month-old male C57/Bl6J mice were anesthetized with isoflurane (induction at 3–4% and maintenance at 1–2%) evaporated in oxygen and administered through a nose mask. The mice were placed on a custom-made stereotaxic frame with a built-in heating bed that maintains body temperature at 37°C. The head was mounted in the stereotaxic frame, the surgical site clipped and cleaned with alternate iodine and ethanol scrubs, and bupivacaine was administered intradermally. A 10-mm midline incision was made over the skull, and the skin and fascia were reflected to allow a 4-mm craniotomy to be bored on the central aspect of the left parietal bone. A Leica Impact One Stereotaxic Impactor device was used to deliver the cortical impact, with an impact velocity of 5.25 m/sec, an impact depth of 1.5 mm, and a dwell time of 0.1 sec. After injury, the incision was closed with staples, anesthesia was terminated, and the animal was placed in a heated cage to maintain normal core temperature for 45 min post-injury. Sham injury consisted of exposure to anesthesia, stereotaxic mounting, skin and fascia reflection, and incision closing with staples.

Drug administration

The γ -secretase inhibitor LY450139 (semagacestat) was dissolved in ethanol and suspended in corn oil. Mice received either 30 mg/kg LY450139 or vehicle (5% ethanol in corn oil) at a final volume of 5 mL/kg by oral gavage. Treatments occurred at 15 min post-injury and 12 h post-injury; $n = 5$ mice for each group.

Golgi staining

For detailed characterization of neuronal processes and spines, we performed Golgi staining using the FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Ellicott City, MD). At 24 h after injury, mice were euthanized and perfused with ice-cold phosphate buffered saline. Brains were immersed in solutions A and B for 2 weeks at room temperature and then transferred into solution C for 48 h at 4°C. The brains were sliced using a Vibratome (VT1000S; Leica, Germany) at a thickness of 150 μ m. Bright-field microscopy images of pyramidal neurons in layers II/III of the cortex, layers II/III of the entorhinal cortex, and granule neurons in the dentate gyrus of the hippocampus were captured using 63x oil-immersion objective on a Zeiss Axioplan 2 (Brighton, MI).

For the layer II/III neurons, we performed two separate counts—dendritic spines on basal shaft (BS) dendrites, and dendritic spines on the apical oblique (AO) dendrites. BS dendrites project directly off the cell soma, and our counts incorporated dendritic spines along a 20 μ m section of the shaft between 30–100 μ m away from soma. Apical oblique (AO) dendrites project off the apical dendrite, and our counts only incorporated primary AO dendrites. We counted spines in a 20 μ m section of the primary AO. Different neurons were used to quantify AO and BS segments of healthy pyramidal neurons of cortical layers II/III. Images were coded, and dendritic spines were counted in a blinded fashion using Image J

Software (National Institutes of Health, Bethesda, MD). From an $n = 5$ mice in each group, we averaged a total of 90 neurons in each group for layer II/III counts, 84 neurons for dentate gyrus counts, and 144 neurons for the entorhinal cortex counts.

A β_{40} enzyme-linked immunosorbent assay (ELISA)

A β_{40} levels were quantified as described previously.²² Briefly, ipsilateral cortex was homogenized in 10 volumes of ice-cold tissue homogenization buffer containing 250 mM sucrose, 20 mM Tris-base, 1 mM ethylenediaminetetraacetic acid and 1 mM ethylene glycol tetraacetic acid (pH 7.4) with mammalian tissue protease inhibitor cocktail. The homogenate was mixed 1:1 with 0.4% diethylamine (DEA), 100 mM NaCl solution using a ground glass pestle in a dounce homogenizer. This mix was centrifuged at 135,000 $\times$ g for 45 min at 4°C. The supernatant was removed (DEA-soluble fraction) and neutralized with 10% 0.5 M Tris-HCl (pH 6.8). Soluble A β_{40} was measured from this fraction using a commercially available ELISA kit (Wako Chemicals, Richmond, VA), according to the manufacturer's instructions.

Statistical analysis

All data were analyzed using a one-way analysis of variance, followed by *post-hoc* analysis with the Newman-Keuls multiple comparison test and presented as the mean $\pm$ standard error of the mean. All statistical tests were performed using GraphPad Prism software, version 5.0d (GraphPad Software, Inc., San Diego, CA), and p values of less than 0.05 were considered statistically significant.

Results

TBI results in a rapid loss of neurons in the cortical area surrounding the primary lesion site

CCI resulted in the development of a considerable lesion in mice, with tissue loss occurring deep into the parietal cortex of the injured hemisphere. In sham-injured mice, Golgi stained neurons had a balanced representation in both hemispheres; however, in the TBI tissue, the injured hemisphere qualitatively appeared to have significantly less neuronal Golgi staining. This was particularly true in the cortical tissue surrounding the lesion site, where a 254 $\pm$ 3.1 μ m ribbon of tissue existed that had almost no Golgi stained neurons. Because our brain tissue was coronal sliced, this neuronal “dead zone” was particularly evident at caudal and rostral edges of the lesion site, even in the absence of a visible lesion (Fig. 1A). Neurons that bordered the dead zone displayed projections that appeared to be retracting away from the lesion site. The dendrites appeared swollen and contained multiple bulbous abnormalities that were visible along the length of the dendrite (Fig. 1B, C).

We have found previously that the CCI model of TBI results in reduced neuronal cells in the hippocampus,¹⁴ and again in this study there were noticeably less Golgi-positive CA1, CA2, and dentate gyrus neurons visible in the ipsilateral hippocampus compared with the contralateral hippocampus of the same mouse (Fig. 1D). While this gave the qualitative impression of less dendritic complexity, on closer examination, it became apparent that the remaining Golgi-stained neurons still appeared relatively normal, with normally shaped dendrites and appreciable dendritic spine counts.

TBI causes a widespread reduction in dendritic spines

Dendritic spine analysis focused on intact neurons in our target areas and did not include neurons that displayed signs of injury such as swelling or axonal abnormalities. Only neurons with appreciable

FIG. 1. Traumatic brain injury results in rapid loss of neurons in the cortical area surrounding the primary lesion site. (A) Representative image of a mouse brain coronal section with Golgi staining at 24h post-injury. A neuronal “dead zone” exists around the lesion where no Golgi stain is taken up into neurons. This is especially evident at the rostral edge of the lesion, marked by an asterisk, where no Golgi stain has been taken up by neurons, but the lesion itself is not present. (B) Image of Golgi-stained neurons with dendrites that appear to be retracting from the dead zone surrounding the lesion. Arrows show dendrites on the left side of the neuron that have swelling and abnormalities associated with dendrite retraction. The dendrites on the side facing away from the dead zone appear to still have dendritic spines and do not show dendritic abnormalities. (C) High magnification image of a dendrite protruding into the dead zone, showing numerous swellings and abnormalities. (D) Representative Golgi-impregnated images of the contralateral and ipsilateral dentate gyrus (DG) of the same mouse. Although there appears to be less dense staining of granule cells in the ipsilateral DG compared with the contralateral DG, the stained neurons do not show overt signs of injury and have dendritic spines.

dendritic spines were included because we did not want to skew our analysis by including neurons that were actively retracting from the lesion site. We sampled from three brain regions in the ipsilateral hemisphere and the corresponding brain regions on the contralateral hemisphere. These sites were (1) layer II/III neurons in the parietal cortex, (2) layer II/III neurons in the entorhinal cortex, and (3) dentate gyrus neurons of the hippocampus. We examined the parietal cortex because this is the brain region closest to the injury site, and the dentate gyrus and entorhinal cortex neurons because they are more distant from the injury site. Neurons from the dentate

gyrus and the entorhinal cortex form the perforant pathway, an important pathway for learning and memory, which can be seriously disrupted after TBI.

In the ipsilateral parietal cortex, we found that TBI caused a 27.5% reduction in the total number of dendritic spines on layer II/III neurons ($p < 0.0001$; Fig. 2A). When broken down by dendritic region, this included a 24% reduction in the number of spines on AO dendrites ($p < 0.001$; Fig. 2B) and a 32% reduction in the number of spines on the BS dendrites ($p < 0.001$; Fig. 2C). Moving away from the primary lesion site, we also found a significant effect of TBI on dendritic spines in the ipsilateral dentate gyrus, with spine number being reduced by 20% compared with sham mice ($p < 0.0001$; Fig. 3A). Dendritic spine loss also occurred in the ipsilateral entorhinal cortex, with TBI causing a 39% spine loss in layer II/III neurons in this brain region, including a 38% reduction in AO and a 40% reduction in BS spines ($p < 0.0001$; Fig. 3B–D).

We also examined the intact, contralateral hemisphere of the brain to assess how a unilateral injury might alter global neuronal structure. Interestingly, CCI caused almost identical reductions in dendritic spine counts in the contralateral hemisphere as in the ipsilateral hemisphere. In the contralateral parietal cortex, there was a 23% reduction in total spines ($p < 0.0001$; Fig. 2A), with a 22% reduction in AO ($p < 0.001$; Fig. 2B) and 25% reduction in BS ($p < 0.001$; Fig. 2C). In the contralateral dentate gyrus, there was a 23% reduction in spines ($p < 0.001$; Fig. 3A), which was similar to

FIG. 2. TBI causes dendritic spine loss in Layer II/III neurons at 24h post-injury. (A) Controlled cortical impact (CCI) causes a reduction in dendritic spines on the pyramidal neurons of cortical layers II/III in both the ipsilateral (ipsi) and contralateral (contra) cortex. (B) Representative Golgi-stained apical oblique (AO) dendrites of pyramidal neurons of layer II/III of the parietal cortex, and a graphic representation of the average spine number for each experimental condition. (C) Representative Golgi-stained basal shaft (BS) dendrites of pyramidal neurons of layer II/III of the parietal cortex, and a graphic representation of the average spine number for each experimental condition. Analysis of variance with Student-Newman-Keuls *post-hoc* test; *** $p < 0.001$ vs. sham. TBI, traumatic brain injury.

the spine loss recorded in the ipsilateral dentate gyrus. Finally, there was a 12% reduction in total spines in the contralateral entorhinal cortex ($p < 0.001$ vs. sham; Fig 3B). While this loss was significant, it was still markedly less than the 39% spine loss recorded in the ipsilateral entorhinal cortex ($p < 0.0001$ vs. ipsilateral entorhinal cortex; Fig 3B), and this result may reflect the distance of the region of interest from the injury epicenter.

These data reveal an acute and widespread loss of excitatory synapses at 24 h post-injury. This spine loss is not restricted to the primary injury site, but instead appears to be a widespread consequence of brain trauma.

The γ -secretase inhibitor LY450139 prevents the TBI-induced increase in A β_{40} , but does not protect against dendritic spine loss after injury

We have shown previously that CCI causes a rapid and significant increase in the production of the Alzheimer disease-related protein A β in mice.^{14,22} Because A β can cause dendritic spine loss *in vitro* and *in vivo*,^{18,19} we wanted to determine the role of A β accumulation on spine loss after TBI.

We exposed a cohort of mice to sham or TBI surgery, and treated them with either vehicle or the γ -secretase inhibitor LY450139. Twenty-four hours post-injury, the mice were euthanized, and levels of A β were measured from the ipsilateral cortex. We found that CCI caused a significant increase in A β_{40} at 24 h post-injury compared with sham controls ($p < 0.001$; Fig. 4). Treatment with LY450139 significantly attenuated this increase in A β after injury ($p < 0.01$; Fig. 4). These data demonstrate that orally administered LY450139 can significantly block A β production in the injured brain.

FIG. 4. Traumatic brain injury-induced A β_{40} accumulation in the ipsilateral cortex is attenuated with the γ -secretase inhibitor LY450139. Controlled cortical impact (CCI) causes an increase in soluble A β_{40} levels at 24 h post-injury that is significantly reduced by treatment with LY450139. A β_{40} levels quantified by enzyme-linked immunosorbent assay. Analysis of variance with Student-Newman-Keuls *post-hoc* test; *** $p < 0.001$ vs. sham.

A second cohort of mice was then exposed to CCI surgery and administered either vehicle or LY450139. At 24 h, the brains were fixed in Golgi solution, and dendritic spine counts from the cortex and hippocampus were conducted. Similar to our first study, we found that TBI caused a significant decrease in dendritic spines in multiple brain regions. In the parietal cortex, we found that TBI caused an 11% reduction in total spine counts in vehicle-treated TBI mice ($p < 0.01$; Fig. 5A), with a 10% reduction in AO ($p < 0.01$; Fig. 5B) and an 11% reduction in BS dendritic spines ($p < 0.01$; Fig. 5C). In the dentate gyrus of vehicle-treated TBI mice, we found a 24% reduction in spine levels compared with vehicle sham mice ($p < 0.001$; Fig. 5D). We found that LY450139 treatment did not significantly affect spine levels in the ipsilateral cortex or hippocampus (Fig. 5A–D), with LY450139-treated TBI mice displaying a similar reduction in dendritic spines as vehicle-treated TBI mice. We also found no significant effect of LY450139 on spine numbers in the contralateral hemisphere (data not shown).

These data demonstrate that TBI-induced dendritic spine loss is independent of A β accumulation after injury.

Discussion

In the present study, we find that CCI injury in mice causes an acute reduction of excitatory synapses, as indicated by a reduction in dendritic spines in multiple regions of the injured brain. Our dendritic spine counts did not include neurons that appeared unhealthy (as indicated by dendritic dystrophy, dendritic blebbing, or atrophic cell bodies), but instead focused on the remaining healthy neurons in the parietal cortex surrounding the lesion site, in the ipsilateral dentate gyrus of the hippocampus, and of the more distal entorhinal cortex. We also included identical sites on the contralateral hemisphere of the brain. Interestingly, we found that TBI caused a similar loss of dendritic spines in both the ipsilateral and

FIG. 5. Traumatic brain injury-induced dendritic spine loss is not attenuated by the γ -secretase inhibitor LY450139. (A) Total dendritic spine counts of layer II/III neurons in the parietal cortex of mice after controlled cortical impact (CCI) and treatment with LY450139. Spine counts on the apical oblique (AO) dendrites (B) and basal shaft (BS) dendrites (C) of layer II/III neurons of the parietal cortex after CCI and treatment with LY450139. (D) Dendritic spine counts from granule cells of the dentate after CCI and treatment with LY450139 treatment. Analysis of variance with Student-Newman-Keuls *post-hoc* test; ** $p < 0.01$; *** $p < 0.001$ vs .sham.

contralateral hemisphere, indicating that the effects of TBI on neurons are not restricted to the lesion site. Finally, we examined the role of γ -secretase and the neurotoxic peptide $A\beta$ on spine loss after injury. We found that a γ -secretase inhibitor LY450139 could prevent the TBI-induced increase in $A\beta$; however, it had no effect on dendritic spine loss after injury. These data demonstrate that spine loss after TBI is independent of injury-induced $A\beta$ accumulation.

Dendritic spine loss after a lateral fluid percussion injury was recently reported in rats.⁸ Campbell and colleagues⁸ reported that 24 h post-TBI, there was a 21% loss in total spines on the AO dendrites of layer II/III cortical neurons, and loss of synaptic activity has also been reported *in vitro*, where stretch injury of hippocampal neurons results in a prolonged loss of excitatory synaptic transmission.²³ In the present study, we found that dendritic spine loss occurred over a wide area after a focal TBI, including a significant loss of dendritic spines in the contralateral cortex and hippocampus. We believe that our extensive loss of excitatory synapse sites is because CCI causes a much more severe injury than that seen with the unilateral fluid percussion injury model. Indeed, the development of a lesion site and the loss of Golgi staining that occurred in our model are not apparent in the fluid percussion study.⁸ The fact that dendritic spine loss has now been reported in two very different models of experimental TBI demonstrates that this may be a common outcome after injury.

One of the major proteins involved in dendritic spine retraction is the calcium/calmodulin dependent phosphatase, calcineurin.²⁴ After lateral fluid percussion injury in rats, calcineurin activity and protein levels of the downstream effector protein cofilin are increased 24 h post-TBI.²⁵ These data suggest that the loss of dendritic spines is caused by the activation of calmodulin and cofilin and the subsequent depolymerization of dendritic actin. Indeed, the loss of dendritic spines in the fluid percussion injury model can be prevented by administration of the calcineurin inhibitor FK506.⁸ Similar to TBI, excess levels of $A\beta$ also cause spine loss mediated by calcineurin, and $A\beta$ -induced spine loss is also sensitive to the calcineurin inhibitor FK506.²⁰ $A\beta$ accumulates very quickly after TBI because $A\beta$ deposits can be identified within as little as 2 h post-injury in the human brain.^{13,15} Further, this accumulation of $A\beta$ also occurs in animal models of TBI,^{12,17} including mouse CCI.^{12,14,22}

In the present study, we again report that CCI causes a significant and rapid accumulation of soluble $A\beta_{40}$ species. This increase in $A\beta$ can almost be completely blocked with the peripheral administration of the γ -secretase inhibitor LY450139. Preventing $A\beta$ accumulation, however, did not impact spine loss after TBI, suggesting that the previously reported effects of TBI on calcineurin are not mediated by the presence of excess $A\beta$. It is important to note that we did not assess the duration of TBI-induced dendritic spine loss in our model, so while we can determine that spine loss is not mediated by $A\beta$ in our present study, it is still possible that reduced $A\beta$ levels after TBI could improve the extracellular microenvironment to enhance spine recovery and reduce functional deficits after injury at later time points.

It is not only the $A\beta$ peptide that is reported to impact dendritic spines, but also γ -secretase itself. The loss of the endoplasmic reticulum from dendritic spines in response to stress has been reported, and treatment with γ -secretase inhibitors can prevent this loss.²⁶ Conversely, a 4-day administration of γ -secretase inhibitors DAPT and LY450139 has been found to reduce spine numbers in mice through an APP dependent pathway.²⁷ Here we found that γ -secretase inhibition did not alter spine loss after TBI, and thus it

does not appear that γ -secretase plays a pivotal role in dendritic spine loss after injury. It is interesting to note, however, that the amount of dendritic spine loss caused by TBI was considerably smaller in the LY450139 study than in our initial experiments. We believe that this could be because of the use of ethanol as a vehicle, because ethanol has been shown previously to have neuroprotective properties.^{28,29} Our study effectively delivered a total of 0.1 g/kg of ethanol to the mice over two administrations. Previous studies have shown that 1 g/kg of ethanol can improve cerebral blood flow and reduce metabolic uncoupling after CCI in mice,²⁹ and 1.5 g/kg can reduce lesion volume and improve outcome in an ischemia model.²⁸ These neuroprotective effects may translate into reduce synapse loss after injury.

In our previous work, we reported a protective effect of the γ -secretase inhibitor DAPT.¹⁴ Here we examined the acute effects of a second inhibitor LY450139, which we would expect to have similar protective effects after TBI as DAPT. While we do not see an effect of γ -secretase inhibition on spine loss, it is difficult to extrapolate the lack of effect of our acute data to the longer-term motor and cognitive benefit we found in a 3-week treatment study. Because our current data clearly show that LY450139 can prevent the acute accumulation of A β after TBI but not dendritic spine loss, however, we can conclude that dendritic spine loss after TBI occurs independently of A β . TBI, including mild TBI, causes calcium release^{30–32} that could result in activation of the calcium/calmodulin dependent proteins. Specifically at the post-synaptic density, the increase in glutamate after TBI could result in activation of NMDA receptors and the influx of Ca²⁺ into the dendritic spine. Thus the loss of dendritic spines may be a neuroprotective event to reduce Ca²⁺ influx and to allow the neuron to regain calcium homeostasis. A two photon imaging study in a mouse model of global ischemia, however, showed that a short ischemic insult could cause a propagating wave of Ca²⁺ to induce dendritic spine remodeling.³³ This wave travelled relatively slowly (3.3 mm/min), and an increase in intracellular calcium occurred simultaneously to the loss of dendritic structure. Interestingly, these data contradict the NMDA-mediated spine loss theory, because the loss of dendritic spines could not be prevented by treatment with MK801.³³

Conclusion

We have demonstrated that a single severe contusion injury can cause the loss of dendritic spines, even in brain regions far removed from the impact site. This loss of dendritic spines was not prevented with the administration of the γ -secretase inhibitor LY450139, despite a significant reduction in TBI-induced A β accumulation. We conclude that dendritic spine loss after TBI is not mediated by increased γ -secretase activity or by the accumulation of A β .

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Author Disclosure Statement

No competing financial interests exist.

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