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SHORT COMMUNICATION

Expression of NgR1-Antagonizing Proteins Decreases with Aging and Cognitive Decline in Rat Hippocampus

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Abstract The myelin-associated inhibitor/Nogo-66 receptor 1 (NgR1) pathway directly functions in negative modulation of structural and electrophysiological synaptic plasticity. A previous study has established an important role of NgR1 pathway signaling in cognitive function, and we have demonstrated that multiple components of this pathway, including ligands, NgR1 co-receptors, and RhoA, are upregulated at the protein level specifically in cognitively impaired, but not age-matched cognitively intact aged rats. Recent studies have identified two novel endogenous NgR1 antagonists, LOTUS and LGI1, and an alternative co-receptor, ADAM22, which act to suppress NgR1 pathway signaling. To determine whether these endogenous NgR1-inhibiting proteins may play a compensatory role in age-related cognitive impairment by counteracting overexpression of NgR1 agonists and co-receptors, we quantified the expression of LOTUS, LGI1, and ADAM22 in hippocampal CA1, CA3 and DG subregions dissected from mature adult and aged rats cognitively phenotyped for spatial learning and memory by Morris water maze testing. We have found that endogenous inhibitors of NgR1 pathway action decrease significantly with aging and cognitive decline and that lower expression levels correlate with declining cognitive ability, particularly in CA1 and CA3. These data suggest that decreased expression of NgR1-antagonizing proteins may exert a combinatorial effect with increased NgR1 signaling pathway components to result in abnormally strong suppression of synaptic plasticity in age-related cognitive impairment.

Keywords Age-related cognitive decline · RhoA · ADAM22 · LGI1 · LOTUS/CRTAC1 · Plasticity · Nogo-66 receptor 1

Introduction

Nogo-66 receptor 1 (NgR1) pathway signaling through RhoA, initiated by binding of myelin-associated inhibitors (MAIs) to NgR1 and mediated by multiple NgR1 co-receptors, suppresses neurite outgrowth and axon regeneration during development and following CNS damage. MAI/NgR1 pathway action also modulates synaptic plasticity in the mature, undamaged CNS by promoting structural rigidity and suppressing functional strengthening of synapses. Anatomical, biochemical, and electrophysiological assessments demonstrate an inverse relationship between MAI/NgR1 pathway expression and hippocampal spine density, efficacy of activity-dependent synaptic plasticity, and spatial learning and memory (Zagrebelsky et al. 2005; Lee et al. 2008; Karlen et al. 2009; Raiker et al. 2010; Delekate et al. 2011). We have previously demonstrated the significant hippocampal upregulation of the MAI ligands Nogo-A, MAG, and OMgp, the NgR1 receptor and its signal-transducing co-receptors in a naturally occurring rat model of human age-related cognitive decline (VanGuilder et al. 2011b, 2012; VanGuilder Starkey et al. 2013) (Supplemental Table 1). Interestingly, induction

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of MAI/NgR1 pathway components occurs specifically in cognitively impaired, but not cognitively intact, aged rats phenotyped for hippocampal cognitive function, and is highly conserved within individual subjects, suggesting an important role of MAI/NgR1-mediated suppression of synaptic plasticity in impaired spatial learning and memory.

The MAI/NgR1 pathway has been well characterized, but recently, endogenous NgR1 antagonists that compete with MAIs for NgR1 binding have been discovered, suggesting an additional level of complexity to NgR1 pathway regulation. Lateral olfactory tract usher substance (LOTUS) is a transmembrane domain-containing secreted protein that antagonizes NgR1 to prevent Nogo-66-mediated growth cone collapse (Sato et al. 2011; Kurihara et al. 2012). Leucine-rich glioma inactivated 1 (LGI1) is a leucine-rich repeat domain-containing secreted protein that competes with Nogo-66 for NgR1 binding, which effectively antagonizes the plasticity-suppressing action of the MAI/NgR1 pathway. Through interaction with ADAM22, a disintegrin and matrix metalloprotease and putative NgR1 co-receptor, LGI1 functions to enhance neuronal outgrowth. The known roles of LOTUS and LGI1 as endogenous NgR1-antagonizing ligands, and ADAM22 as an NgR1-interacting surface receptor, suggest a potential mechanism that may compensate for abnormal induction of MAI/NgR1 signaling in age-related cognitive decline (Fig. 1). The goal of the present study was to determine whether hippocampal expression of LOTUS, LGI1 and ADAM22 is regulated with cognitive impairment and to determine their potential relationship to spatial learning and memory ability.

Materials and Methods

Animals: Behavior and Sample Preparation

Behavioral stratification of subjects and dissection of CA1, CA3, and DG subregions has been described in detail elsewhere (VanGuilder et al. 2011a, 2012; VanGuilder Starkey et al. 2012, 2013.) All animal experiments were performed in accordance with IACUC and AALAC approved procedures. In brief, mature adult (12 months) and aged (26 months) male Fischer 344 × Brown Norway (F1) hybrid rats were purchased from the National Institute on Aging rodent colony maintained by Harlan Industries (Indianapolis, IN) and housed singly in the OUHSC Reynolds Oklahoma Center on Aging barrier facility, with water and food (Purina Mills, Richmond, IN) freely available. Rats were tested for hippocampal spatial learning and memory ability by Morris water maze, and aged rats were classified as cognitively intact or impaired relative to the mature adult group based on mean proximity to the escape platform location during probe trials (VanGuilder et al. 2011a, 2012; VanGuilder Starkey et al. 2012, 2013).

Rats were sacrificed by decapitation without anesthesia 1 week after conclusion of behavioral testing. CA1, CA3, and DG subregions were isolated individually from the left and right hippocampi as previously described (Newton et al. 2005; VanGuilder et al. 2011a, 2011b; VanGuilder Starkey et al. 2013). Accuracy of our dissections has been verified by quantifying expression of subregion-specific genes (described in VanGuilder Starkey et al. 2013).

Immunoblotting

As previously described (VanGuilder et al. 2012; VanGuilder Starkey et al. 2012), soluble protein from hippocampal CA1, CA3, and DG was extracted by homogenizing samples in 1 % Tween-20, 100 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, and cOmplete Mini EDTA-free protease inhibitor tablets (Roche, Indianapolis, IN). Soluble protein concentrations were determined by BCA assay (Pierce, Rockford, IL), and samples were diluted in lysis buffer and LDS sample buffer (Life Technologies, Carlsbad, CA) and denatured/reduced with 50 mM dithiothreitol at 95 °C. Sample sizes were as follows: CA1: adult $n = 7$, aged intact $n = 7$, aged impaired $n = 10$; CA3: adult $n = 7$, aged intact $n = 7$, aged impaired $n = 10$; DG: adult: $n = 5$, aged intact: $n = 5$, aged impaired: $n = 6$. Prepared samples were separated using 26-well Criterion TGX 4–20 % acrylamide gradient gels (BioRad Hercules, CA) transferred to PVDF (GE Healthcare, Piscataway, NJ) and blocked with 3 % bovine serum albumin in PBS/1 % Tween-20. Membranes were probed with goat polyclonal primary antibodies (anti-LOTUS: sc160254, anti-LGI1: sc9581, anti-ADAM22: sc25999; Santa Cruz Biotechnology, Santa Cruz, CA), washed in PBS/1 % Tween-20, incubated with HRP-conjugated mouse anti-goat secondary antibody (sc2768, Santa Cruz Biotechnology), developed with enhanced chemiluminescent substrate, visualized on film, and digitized at 800 dpi for quantitation by semi-automated digital densitometry (ImageQuant TL, GE Healthcare). Immunoreactive bands were eliminated by incubation of the primary antibody with their respective blocking peptide (5:1 w/w) (LOTUS: sc160254 P, LGI1: sc9581 P, ADAM22: sc25999 P) for 2 h before addition to the blot (data not shown). Immunoblot data for each sample were normalized to corresponding whole-lane densitometry of a total protein-stained gel (VanGuilder et al. 2011a, 2011b, 2012). No differences in protein banding, as determined by protein staining gels, or Actin immunoreactivity were observed, as described previously (VanGuilder et al. 2012).

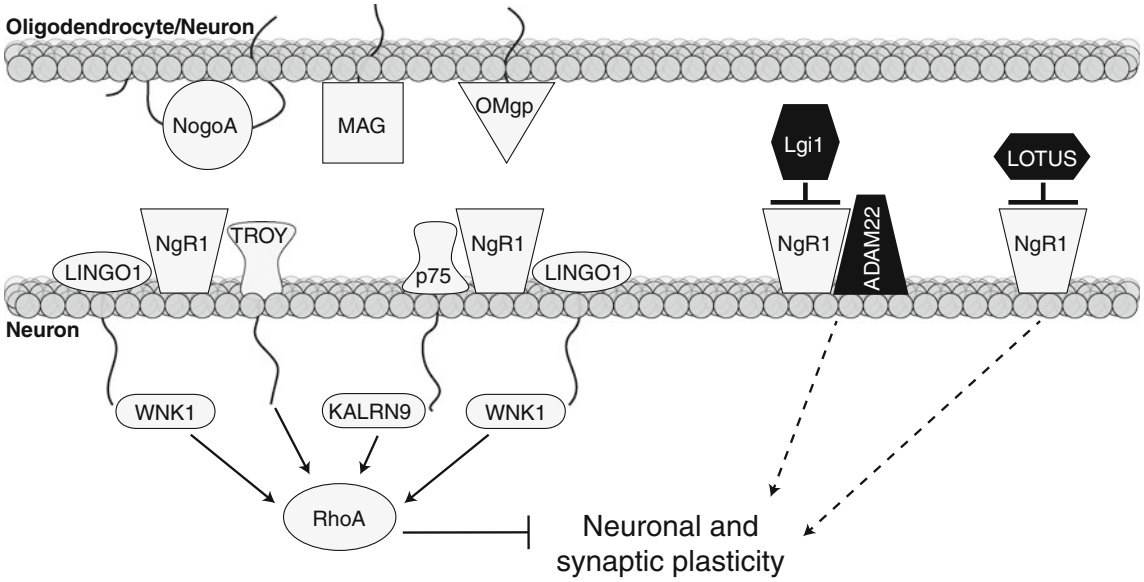

Fig. 1 LOTUS, LGI1, and ADAM22 antagonize MAI/NgR1-mediated inhibition of plasticity. The plasticity-suppressing ligands Nogo-A, MAG, and OMgp bind to a common receptor, NgR1. Two co-receptor complexes (NgR1/LINGO-1/TROY and NgR1/LINGO-1/p75) transduce MAI/NgR1 signals through intermediaries to activate the GTPase RhoA, which activates a cascade of plasticity-suppressing effectors and resulting in decreased structural remodeling and functional strengthening of synapses. The newly discovered NgR1 antagonists LOTUS and LGI1 compete with MAIs for NgR1 binding sites and inhibit MAI/NgR1 pathway-mediated suppression of plasticity. ADAM22 interacts with NgR1 to create an LGI1-binding moiety that facilitates LGI1 antagonism of NgR1 to promote plasticity

qPCR

Quantitation of gene expression was conducted as previously described (Bixler et al. 2011; VanGuilder Starkey et al. 2012) using RNA isolated and purified using standard phase separation/isopropanol precipitation and the RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized using the High-capacity cDNA Reverse Transcription kit (Life Technologies), and qPCR was performed using TaqMan gene-specific primer/probe assays (LOTUS:Rn00592920_m1, LGI1:Rn01454071_m1, ADAM22: Rn01526955_m1; Life Technologies) with a 7900HT Sequence Detection System (Life Technologies), with β-actin (Rn00667869_m1) as the endogenous control. Sample sizes were as follows: CA1: adult $n = 7$, aged intact $n = 7$, aged impaired $n = 10$; CA3: adult $n = 7$, aged intact $n = 7$, aged impaired $n = 10$; DG: adult: $n = 7$, aged intact: $n = 7$, aged impaired: $n = 10$. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with automatic thresholding (SDS 2.2.2 software, Life Technologies).

Statistical Analysis

Morris water maze behavioral testing data, including statistical analysis of group acquisition and probe trial data, have been extensively described elsewhere (VanGuilder et al. 2011a, 2011b, 2012; VanGuilder Starkey et al. 2012, 2013) and are included to provide context for interpretation of protein data. Protein and mRNA data were evaluated by one-way ANOVA with Student Newman Keuls post hoc testing (ANOVA/SNK). Relationships between protein expression and water maze performance (mean proximity to platform location) were performed by Pearson product moment correlation analysis.

Results

Decreased expression of LOTUS, LGI1, and ADAM22 with advanced aging was observed throughout the hippocampus and was frequently exacerbated in aged rats with cognitive deficits (Fig. 2). Significant reductions in LGI1 were evident in CA1, CA3, and DG of aged impaired rats compared to mature adults and ranged from 30 % in CA1 ($p < 0.01$) to greater than 50 % in CA3 ($p < 0.001$) and DG ($p < 0.05$). LGI1 expression in aged impaired rats was also significantly reduced compared to aged intact rats in CA1 ($p < 0.05$) and CA3 ($p < 0.001$), but not DG. LOTUS followed a similar trend, albeit to a lesser magnitude, and reached statistical significance in CA3 with significantly less LOTUS expression in aged impaired rats compared to aged intact and adult rats ($p < 0.05$ and $p < 0.01$, respectively). Significantly decreased ADAM22 expression was

evident in CA1 of both the aged intact and aged impaired groups compared to adults (~20 %, $p < 0.05$). In CA3, ADAM22 was reduced by 35 % in aged intact rats ($p < 0.01$) and by nearly 60 % in aged impaired rats ($p < 0.001$) compared to adults. In DG, a 30 % reduction in ADAM22 was observed in aged impaired rats ($p < 0.05$). Cognitive status-dependent decreases (i.e., aged impaired vs. aged intact) in NgR1-antagonizing protein expression were observed in CA1 (LGI1), CA3 (LGI1, LOTUS), but not DG, while a combinatorial effect of aging and cognitive impairment (aged impaired vs. adult) was observed more frequently [CA1 (LGI1, ADAM22), CA3 (LGI1, LOTUS, ADAM22), and DG (LGI1, ADAM22)]. These decreases were evident in the same samples for which cognitive status-dependent increases in MAI ligands (Nogo-A, MAG, and OMgp), NgR1 receptor, and co-receptors (TROY, LINGO-1, p75) were evident (Supplemental Table 1) (VanGuilder et al. 2011b, 2012; VanGuilder Starkey et al. 2013).

We also observed an inverse relationship between protein expression and Morris water maze performance (Fig. 3). Decreasing spatial cognitive ability (i.e., higher mean proximity values) correlated significantly with LGI1 expression in CA1 ($r = -0.51$, $p < 0.05$) and CA3 ($r = -0.69$, $p < 0.001$) and with LOTUS expression in CA3 ($r = -0.051$, $p < 0.05$). ADAM22 expression was consistently negatively correlated with spatial cognition in all three subregions assessed (CA1: $r = -0.44$, $p < -0.05$; CA3: $r = -0.60$, $p < 0.01$; DG: $r = -0.61$, $p < 0.05$).

Interestingly, protein expression changes were recapitulated at the level of LOTUS, LGI1, and ADAM22 mRNA only in CA1 and DG, but not CA3 (Figure S1).

Discussion

We previously identified the plasticity-suppressing MAI/NgR1 pathway as upregulated in the hippocampus specifically in aged rats with deficits of spatial learning and memory (VanGuilder et al. 2011b, 2012; VanGuilder Starkey et al. 2013). LOTUS and LGI1 are recently identified endogenous NgR1 antagonists that compete with MAIs for NgR1 binding, while ADAM22 interacts with NgR1 to facilitate LGI1/NgR1 binding (Thomas et al. 2010). The findings of our current study indicate that hippocampal expression of LOTUS, LGI1, and ADAM22 is significantly depleted from CA1, CA3, and DG subregions in aged, cognitively impaired rats and identified a strong correlation of decreasing protein expression with decreasing cognitive ability. Interestingly, while decreased expression of these proteins was frequently observed in aged cognitively intact rats, this occurred to a lesser magnitude in all cases. Together, these data suggest that an endogenous mechanism of NgR1 pathway inactivation fails to compensate for NgR1 pathway induction in the hippocampus with age-related cognitive decline.

The proteins investigated here also function to modulate neuronal function in a variety of ways independent of NgR1 interaction. In addition to its known roles in epilepsy (Fukata et al. 2010), hippocampal LGI1 mediates postnatal pruning and functional maturation of glutamatergic synapses and facilitates AMPA receptor-mediated synaptic transmission (Zhou et al. 2009; Fukata et al. 2010). Interestingly, LGI1, which is highly enriched in CA3 pyramidal and DG granule layers (Herranz-Perez et al. 2010) and transcribed almost exclusively by neurons (Senechal et al. 2005), forms a tripartite complex

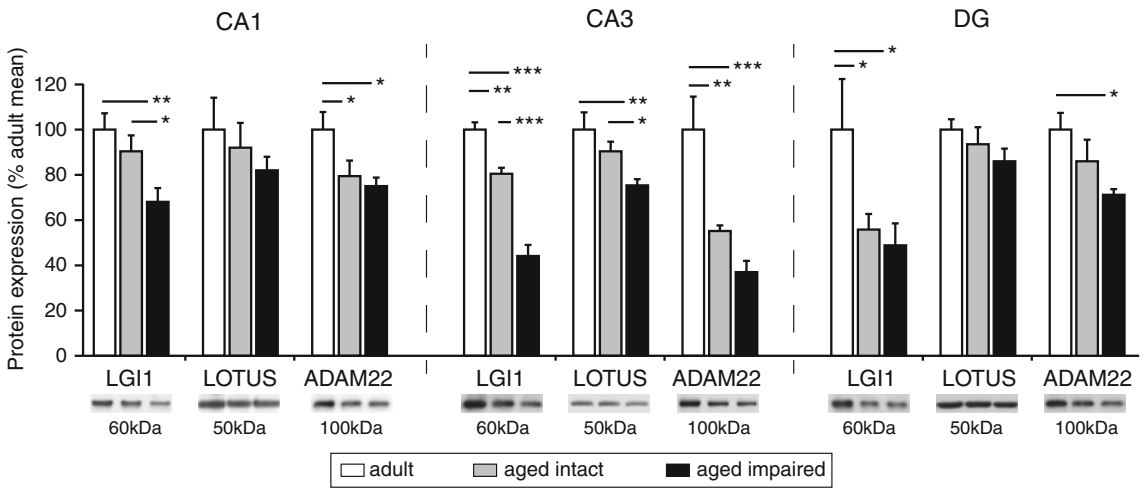

Fig. 2 Expression of NgR1 antagonists decreases with aging and cognitive decline protein expression of the antagonist ligands LOTUS and LGI1 and the NgR1 co-receptor ADAM22 decreases significantly in CA1, CA3, and DG subregions with advanced aging. Additional significant decreases were evident specifically between aged intact and aged impaired rats in CA1. Data are presented as % adult mean ± standard error; representative *blot images* are included as *insets*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.05$; ANOVA/SNK

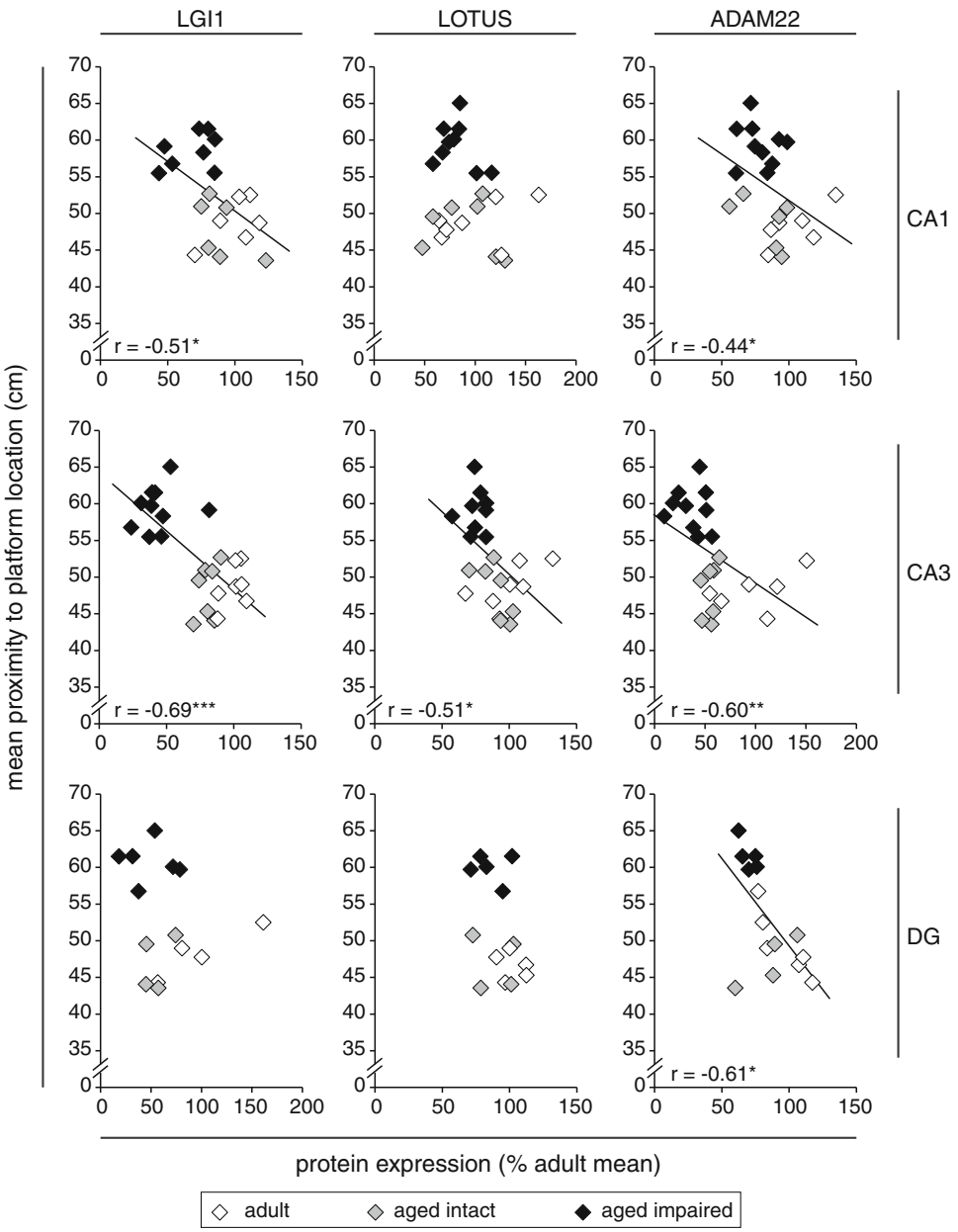

Fig. 3 Expression of NgR1 antagonists correlates with spatial learning and memory ability. There is a significant relationship between protein expression of LOTUS, LGI1 and ADAM22 and mean proximity to the escape platform location during Morris water maze probe trials of spatial learning and memory. Lower protein expression was associated with greater proximity values (i.e., indicating inferior targeting of the escape area), particularly in CA3. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; Pearson correlation

including ADAM22 and PSD95 (Ogawa et al. 2010) in which ADAM22 is localized to synapses by LGI1 and anchored by PSD95, where it may modulate AMPA receptor aggregation through interactions with stargazin (Fukata et al. 2006). ADAM22 itself has profound effects on dendritic maturation and clustering of potassium channels at axonal juxtaparanodes and initial segments (Zhou et al. 2009; Ogawa et al. 2010). LOTUS, on the other hand, impacts gross axonal morphology and fasciculation (Sato et al. 2011) and influences activity-dependent plasticity predominantly through its antagonism of NgR1. It is likely that concomitant upregulation of MAI/NgR1 signaling components and downregulation of NgR1 antagonists exert a combinatorial effect on hippocampal synapses, leading to aberrant neurotransmission and abnormal inhibition of synaptic plasticity which is reflected in impaired spatial learning and memory (Fig. 1).

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Conflict of interest The authors have no conflicts of interest to declare.

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