Expression profiles of the Tau-associated genes *GSk3β*, *CAPN1*, and *CDK5R1* in the brain cortex of aged female cynomolgus monkeys with cognitive impairment

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ABSTRACT

Background: Alzheimer's disease (AD) is characterized by the buildup and aggregation of misfolded proteins in the brain, including amyloid-β (Aβ) and hyperphosphorylated tau. The hyperphosphorylation state of Tau protein plays an important role in the development of AD. Our previous studies developed and characterized the cynomolgus monkey as a spontaneous animal model of AD.

Aim: We demonstrated the validity of the model through experimental investigations of the relationship between cognitive decline and AD neuropathy. There is, however, little information about the expression of hyperphosphorylated tau-related genes in various brain areas in the cynomolgus monkey spontaneous AD model.

Methods: In the present study, total RNA was extracted from archived cortex and hippocampus tissues from the brains of two groups of cynomolgus monkeys, adult (10–12 years old, $n = 5$) and aged (> 20 years old, $n = 4$). The expression of the tau-protein-associated genes kinase 3 beta, calpain 1, and cyclin-dependent kinase 5 regulatory subunit 1 was evaluated using RT-qPCR.

Results: The expression of all three genes increased by up to fivefold in the cortical brain area of aged subjects compared with adults.

Conclusion: Our results add weight to the utility of cynomolgus macaques as a valid spontaneous model in translational preclinical research involving studies of the effect of aging on the formation of hyperphosphorylated tau protein, which causes AD-related lesions in the brain.

Keywords: Cynomolgus monkey, Hyperphosphorylated tau, Gene expression, Aging, Alzheimer.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the buildup and aggregation of misfolded proteins in the brain, including amyloid-β (Aβ) and hyperphosphorylated tau (Jellinger, 1998). More than 55 million people worldwide are living with AD, and this number is expected to nearly double every 20 years. The Centers for Disease Control and Prevention reported that the number of people living with AD doubles every 5 years beyond the age of 65 (Alzheimer's Disease Association, 2024).

Animal models are important tools for the study of AD and in the preclinical testing of potential drug candidates (Chen and Zhang, 2022). Non-human primates (NHPs) are particularly relevant as reliable animal models of complex diseases like AD with respect to the etiology and treatment of the disease (Darusman *et al*., 2021; Jiang *et al*., 2024). We found that a proportion of aged cynomolgus monkeys (*Macaca fascicularis*, long-tailed macaques) spontaneously develop dementia resembling human AD with respect to memory loss and lesions in the brain, including the build-up of amyloid beta$_{1-42}$ and phosphorylated tau threonine 231 (Darusman *et al*., 2014a).

Cynomolgus monkeys have a similar amyloid beta 42 (Aβ$_{42}$) as humans, and cerebral amyloid angiopathy

was observed in aged monkeys. In contrast to humans, amyloids were found to deposit in the small veins and capillaries. Phosphorylated tau was found intracellularly in the neurons, indicating a possibility of an early stage of the formation of tangles (Darusman *et al*., 2014b). Other research has revealed similar qualitative disease sequences and patterns in aged Rhesus monkeys (*Macaca mulatta*) that are similar to those in humans (Paspalas *et al*., 2018).

Hyperphosphorylation of tau protein results in the formation of neurofibrillary tangles. Tau proteins are hyperphosphorylated as a result of changes in kinase and phosphate activity, which can result in neurofibrillary tangles in brain cells and ultimately cell death (Holper *et al.*, 2022). Hyperphosphorylated tau protein is a result of defective cellular signaling, and tau becomes abnormally hyperphosphorylated, which eventually leads to microtubule disassembling and the combination of free tau molecules into paired helical filaments (Yang *et al.*, 2024).

Natural tauopathy in monkeys older than 30 years of age resembles neurodegeneration or the advanced stages of human brain aging (Stonebarger *et al.*, 2021). We recently (Rosmanah *et al.*, 2024) analyzed the mRNA expression of the genes *Presenilin2* (*PSEN2*) and A *Disintegrin and Metalloproteinase 10* (*ADAM10*) in the aged cynomolgus monkey brain (Rosmanah *et al*., 2024). These genes are responsible for the amyloid precursor protein sequence of proteolytic cleavage and the subsequent formation of amyloid β peptides. *PSEN2* expression was higher in the adult control group and ADAM10 expression was lower in the aged group, similar to that found in human AD patients (Kabir *et al*., 2020; Lipper *et al*., 2023).

In the present study, we examined the expression of three genes that play a role in the processing of tau protein in the brain using RT-qPCR to evaluate the quantitative expression of glycogen synthase kinase 3 beta (*GSK3β*), calpain 1 (*CAPN1*), and cyclin-dependent kinase 5 regulatory subunit 1 (p35) (*CDK5R1*) with the aim to further consolidate the cynomolgus monkey as a high-validity animal model of human AD.

Material and Methods

Subject and sample collection
From previous studies, we selected brain tissue from our archives. Four female cynomolgus monkeys aged >20 years met the requirements of low levels of $A\beta_{42}$ (<5000 pg/ml) and low Delayed Response Task (DRT) performance (<40%) for the memory-affected group (Darusman *et al*., 2014a, b). Five adult female subjects, 10–12 years of age, characterized by high-circulating $A\beta_{42}$ and high DRT performance were selected as the control group. Subject age was determined from birth certificates for animals born in captivity and from dental scaling (Swindler, 2002) and the estimated year of birth for animals born in the wild. All subjects were housed at the AAALAC-accredited Primate Research

Center, Bogor Agricultural University (PRC, IPB) in pairs or social groups of various sizes.

Sample collection and project license
This study examined brain tissue from our archives from nine female cynomolgus macaques, which were separated into two groups: four aged (≥ 20 years) and five adults (ages 10–12 years). Brain tissue samples from the cortex and hippocampus were analyzed. Project authorization was granted by KPKPHP4 (Commission for Supervision of Animal Welfare in Research, Testing, Education, and Captivity) after an ethical cost-benefit analysis of the use of animals for this project (IPB number PRC-19-A012).

RNA extraction and synthesis of cDNA
The brain cortex and hippocampus, 2–3 mm sections, were extracted for total RNA using RNeasy Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's guidelines. A Nanodrop 1000 spectrophotometer was used to measure the amount of RNA (Thermofisher Scientific, USA). Reverse transcriptase was used for cDNA synthesis following standard operating procedures (Sensifast cDNA Synthesis Kit, Bioline, Meridian Bioscience, USA).

RT-qPCR amplification
A CFX Opus 96 instrument was used to perform the PCR amplification process (Biorad, USA). The following formulation was used for each reaction: 2 μl of cDNA as a template, 18 μl of a mixture containing 6 μl of nucleotide-free water, 10 μl of Sensifast Sybr mix (Bioline, Meridian Bioscience, USA), and 1 μl of forward and reverse primers for *CAPN1*, *CDK5R1*, GSK3, and GAPDH as reference genes. The nucleotide sequences of these primers are presented in Table 1 (Park *et al*., 2015). The following steps were performed in the qPCR amplification program: pre-denaturation at 95°C for 2 minutes, denaturation at 95°C for 10 seconds, annealing at 55°C for 20 seconds, and extension at 65°C for 10 seconds. This cycle was repeated 40 times.

Data analysis
SPSS version 26 and Microsoft Excel were used to analyze the data. The Shapiro–Wilk test and the Levene test were used to determine whether the data were normal or homogeneous. Then, an independent *t*-test was performed to check for variations across areas and age groups. Cycle threshold (Ct) data from qPCR is processed with the 2-Ct formula to obtain the relative quantification (RQ) result. mRNA expression levels in fold-change were determined using the RQ value after normalization to the *GAPDH* housekeeping gene. P-levels < 0.05 were considered significant.

Results
The qPCR results demonstrated differences in the mRNA expression of *CAPN1, CDK5R1,* and *GSK3* between adult and aged cynomolgus monkeys (Figs 1–3). There was a significantly higher expression of *CAPN1, CDK5R1, and GSK3* mRNA in the cortex

**Table 1.** The primers used in this study were modified from those used by Park *et al.* (2015).

Gene symbol	Gene name	Primer forward (F)/Reverse (R)
CDK5RI	Cyclin-dependent kinase 5 regulatory subunit 1	F:GCTGCCTTGGAAGAGAATCG R: GTGCGTGATGTTGTTCTGGT
CAPN1	Calpain 1, (mu/I) large subunit	F: ATGACCAGATCCAGGCCAAT R: CTCCTTCACGCTGATCTCCA
GSK3β	Glycogen synthase kinase 3 beta	F: TCGCCATCAAGAAAGTATTGCA R: CGCAATCGGACTATGTTACAGT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACAACAGCCTCAAGATCGTCAG R: ACTGTGGT/CATGAGTCCTTCC
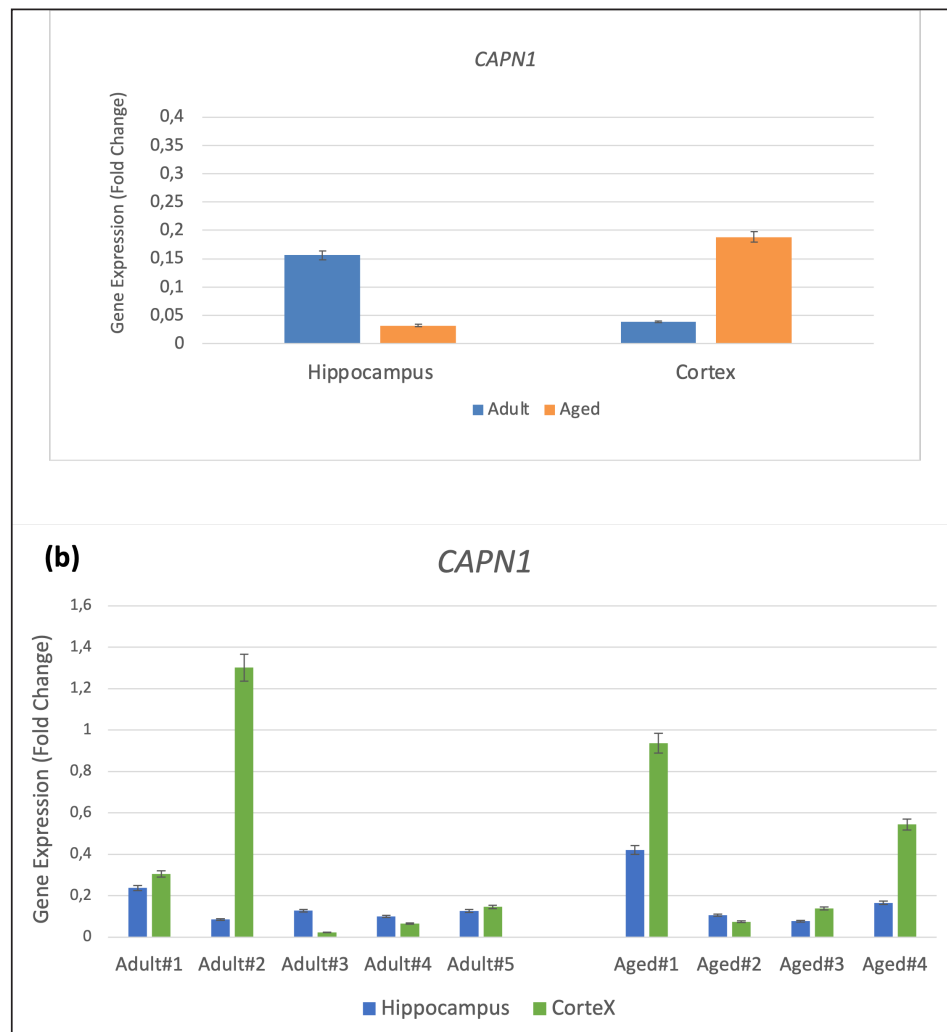
Fig. 1. Gene expression of *CAPN1* in the hippocampus and cortex of adult and aged cynomolgus monkeys (a) Group gene expression (b) Individual gene expression. Values are presented as means error bars = SEM.

tissue of aged animals compared with the cortex tissue of the adults. The expression of CAPN1 was higher in older adult's cortical region than in adult's cortex region. In addition, expression in the hippocampus
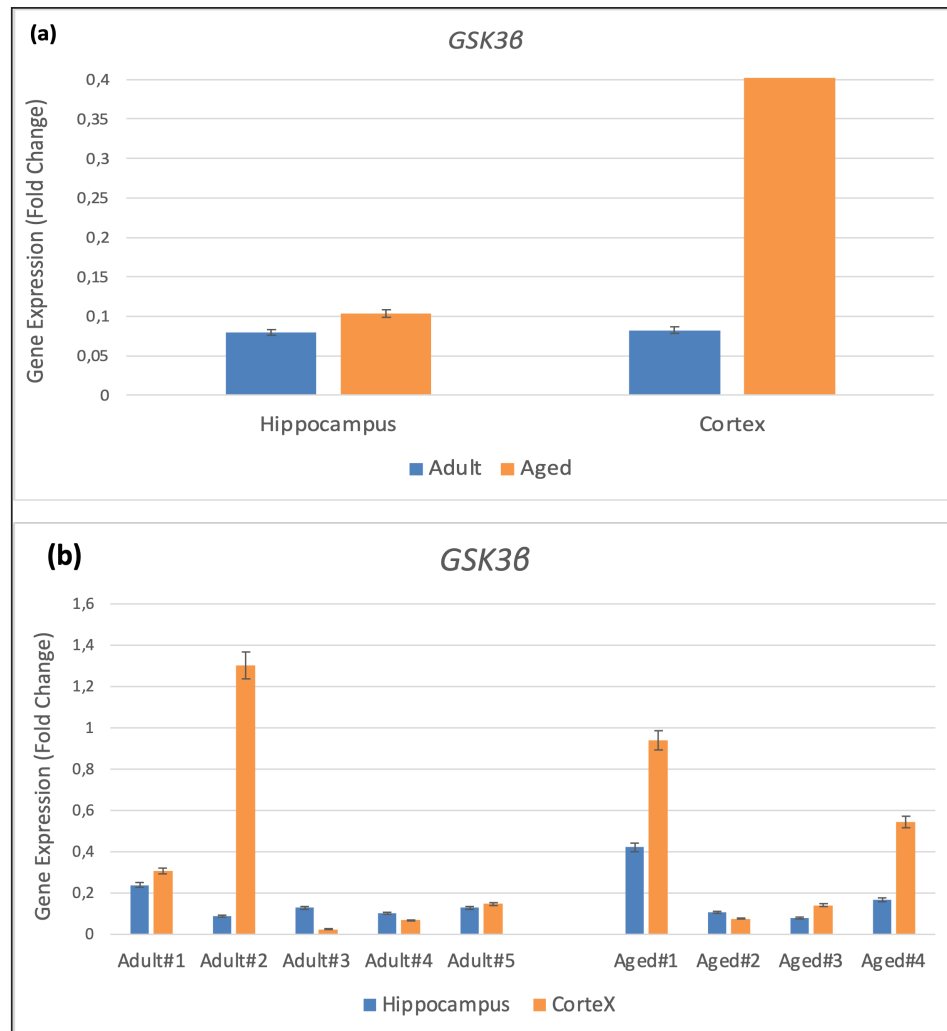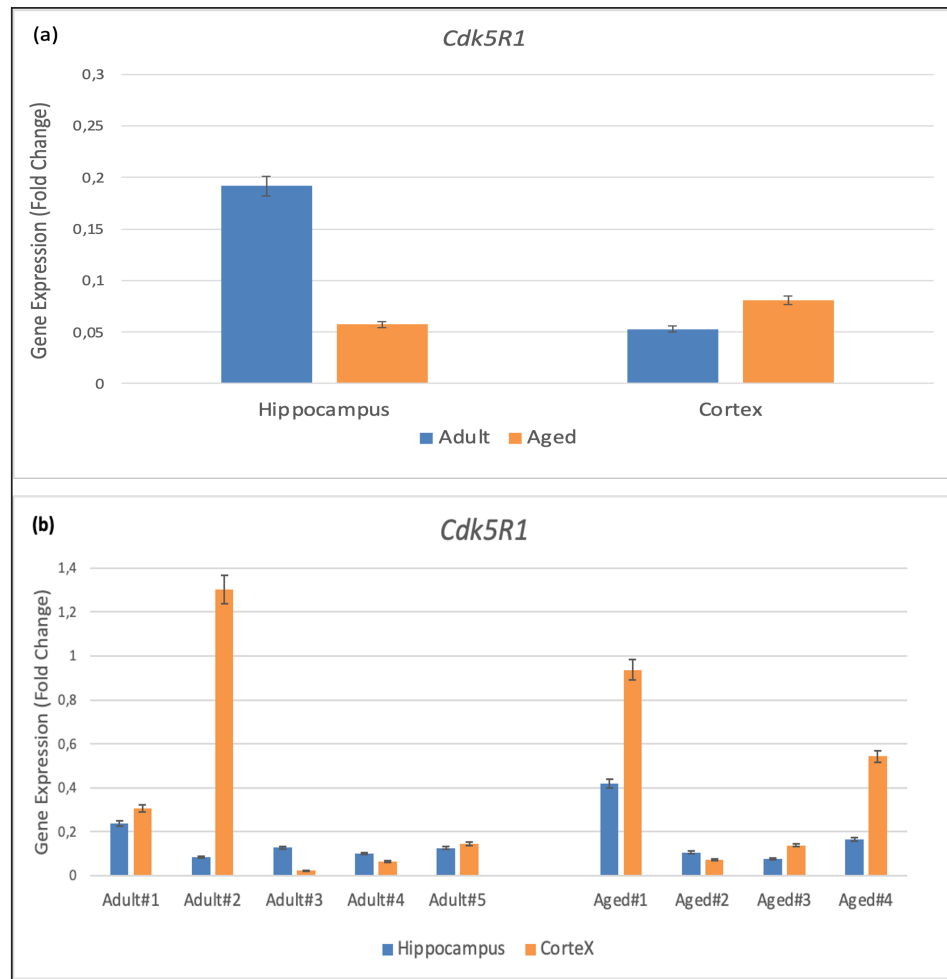
Fig. 2. Gene expression of *GSK3β* in brain hippocampus and cortex of adult monkeys compared with aged monkeys. (a) Group gene expression. (b) Individual gene expression. Values are presented as means error bars = SEM.

region was 4.9-fold higher in adults than in aged monkeys. The *GSK3β* gene expression in older adults' cortex and hippocampus was significantly higher than in the animals in the adult group. The aged cortex expression was 5.9-fold higher and the hippocampus expression was 1.3-fold higher in the aged group than in the adult group. The *CDK5 expression of CDK5R1* older adults' cortex was 1.5-fold higher than that in the adult monkeys. Meanwhile, the adult hippocampus had a 3.3-fold higher expression compared to monkeys in the older adults group.

Discussion

The accumulation of phosphorylated tau proteins in neurofibrillary tangles in the brain is closely linked to neuronal loss and cognitive function in AD. Tau phosphorylation is regulated by genes including *CAPN1*, *CDK5R1*, and *GSK3β* (Park *et al.*, 2015; Spreafico *et al.*, 2018; Lauretti and Dincer, 2021), and in the present study, the cortical regions of older monkeys (aged) exhibited higher levels of gene expression compared with the cortical regions of younger monkeys (adult). The aging process involves a complex interplay of genetic, epigenetic, and environmental factors that lead to changes in gene expression. The increased expression of *CAPN1, CDK5R1,* and *GSK3β* may be part of a broader pattern of altered gene regulation in aging neurons. These changes could be driven by factors such as increased inflammatory signals, altered growth factor levels, and changes in cellular metabolism. Neurons become more susceptible to damage and degeneration as they age. The increased expression of these genes may be associated with early neurodegenerative changes, even

Fig. 3. Gene expression of *Cdk5R1* in the hippocampus and cortex of adult cynomolgus monkeys compared with aged monkeys. (a) Group gene expression. (b) Individual gene expression. Values are presented as means error bars = SEM.

in the absence of overt disease. This upregulation may reflect an attempt to cope with accumulating damage, but it may also contribute to a pathological cycle of tau phosphorylation and neuronal dysfunction.

In addition to playing a physiological role in neuroprotection and synaptic plasticity in the mammalian brain, *CAPN1* is involved in the proteolysis of tau kinases like *GSK3β* and *CDK5R1* (Kurbatskaya *et al.*, 2016; Mahaman *et al.*, 2019; Su *et al.*, 2020). The regulation of the activation of calpains, which are involved in numerous cell life processes, is impaired with advancing age, elevated oxidative stress, and other excitotoxicity. The increased activation of these proteases implicates their involvement in the pathogenesis of several illnesses, including neurodegenerative diseases like AD (Mahaman *et al.*, 2019). According to the findings of this study, older cynomolgus monkeys had greater *CAPN1* gene expression levels of *CAPN1* in the cortex than adults

did. This is due to the possibility that numerous physiological processes, such as increased oxidative stress, have taken in older adults cynomolgus monkeys, which may have an impact on the activation increase. Aging is associated with increased oxidative stress and calcium dysregulation, which can activate the synthesis of *CAPN1*. Higher expression of *CAPN1* can lead to increased cleavage of various substrates, including the CDK5 activators p35 to p25, which can then abnormally activate *CDK5*. This response may be a response to increased cellular damage or an attempt to manage protein aggregates common in aging brains. The GSK3 kinase that eliminates Ser21/9, *GSK3β*, can be activated by calpain at its N-terminal portion (Hoffmeister *et al.*, 2020). According to the findings indicating an increase in calpain and *GSK3β* activity in the cortex region of aged animals, there is a positive link between *CAPN1* and *GSK3β* in the brains of elderly cynomolgus monkeys and calpain-

mediated proteolysis. Numerous players participate in neurogenerative processes, such as *GSK3β*'s cleavage and tau phosphorylation. Calpain 1 may be responsible for the up-regulation of *GSK3β*, abnormal hyperphosphorylation of tau, and neurofibrillary degeneration in AD (Jin *et al.*, 2015). Therefore, it seems likely that in aged cynomolgus monkeys, tau phosphorylation occurs as a result of gradually increasing *GSK3β* expression, which confirms the findings of Lauretti and co-workers that *GSK3β* expression level increases with advancing age (Lauretti *et al.*, 2020).

The key component of the helical filament pairs is the phosphorylated tau protein, which can possibly lead to the development of paired helix filaments if *GSK3β* levels rise (Rankin *et al.*, 2007). The *GSK3β* gene is involved in a variety of activities, including hyperphosphorylation, memory loss, increased amyloid-beta formation, inflammation, and apoptosis mediators, directly causing the loss of neurons in AD (Rankin *et al.*, 2007).

CDK5R1 is a CDK5/p25 activator that may play a role in AD-related nerve degeneration. The protease calpain converts p35/*CDK5R1* to CDK5/p25, which may have a detrimental effect on AD by activating *CDK5* and tau phosphorylation (Rankin *et al.*, 2007). Older cynomolgus monkeys have higher *CAPN1* and *CDK5R1* gene expression in the cortex than do younger monkeys. Accumulation of p25/CDK5, which may have a role in the etiology of cytoskeletal abnormalities and neuronal death in neurodegenerative illnesses, has been reported after increased expression of the *CDK5R1*/p35 gen (Cooper-Patrick *et al.*, 2024).

It has been suggested that as long as NHPs are allowed to age naturally, they are somewhat resistant to the development of tau pathology (Jester *et al.*, 2022). However, the findings of the present study demonstrate that gene expression in aged cynomolgus monkeys (>20 years) is increased in the cortex region for the three genes we examined. Research utilizing different gene markers and cynomolgus monkeys older than 30 years is required to determine whether other regulatory genes are involved in the creation of neurofibrillary tangles.

Although this study successfully utilized qRT-PCR methods to analyze the expression of *CAPN1, CDK5R1*, and *GSK3β* in the cortex of aged and adult monkeys, it was limited by the lack of protein-level validation and functional assays to directly correlate gene expression changes with tau phosphorylation and neuronal health. The protein expression analysis and functional experiments is needed for the future study to provide a comprehensive understanding of the molecular mechanisms driving these changes in the aging brain.

Conclusion

In summary, the higher expression of *CAPN1, CDK5R1,* and *GSK3β* in the cortex of aged monkeys suggests a complex interplay between aging, cellular stress responses, and potential early neurodegenerative changes. These gene expression changes may contribute to altered tau phosphorylation and other protein modifications that influence neuronal health and function in the aging brain. Further studies are needed to elucidate the precise mechanisms and consequences of these changes, which could provide new insights into the molecular understanding of brain aging and neurodegenerative diseases.

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