Cholinergic Involvement in Alzheimer's Disease. A Link with NGF Maturation and Degradation

A. Claudio Cuello · Martin A. Bruno · Simon Allard · Wanda Leon · M. Florencia Iulita

Abstract Basal forebrain cholinergic neurons are highly dependent on nerve growth factor (NGF) supply for the maintenance of their cholinergic phenotype as well as their cholinergic synaptic integrity. The precursor form of NGF, proNGF, abounds in the CNS and is highly elevated in Alzheimer's disease. In order to obtain a deeper understanding of the NGF biology in the CNS, we have performed a series of ex vivo and in vivo investigations to elucidate the mechanisms of release, maturation and degradation of this neurotrophin. In this short review, we describe this NGF metabolic pathway, its significance for the maintenance of basal cholinergic neurons, and its dysregulation in Alzheimer's disease. We are proposing that the conversion of proNGF to mature NGF occurs in the extracellular space by the coordinated action of zymogens, convertases, and endogenous regulators, which are released in the extracellular space in an activity-dependent fashion. We further discuss our findings of a diminished conversion of the NGF precursor molecule to its mature form in Alzheimer's disease as well as an augmented degradation of mature NGF. These combined effects on NGF metabolism would explain the well-known cholinergic atrophy found in Alzheimer's disease and would offer new therapeutic opportunities aimed at correcting the NGF dysmetabolism along with Aβ-induced inflammatory responses.

Keywords NGF · Alzheimer's disease · Basal forebrain cholinergic neurons · Plasmin · MMP-9

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Introduction

The cholinergic involvement in the Alzheimer's disease (AD) pathology is well established. A significant loss of neurochemical cholinergic markers in the cerebral cortex of AD brains was reported in the 1970s by Davis and collaborators, and Bowen and collaborators (Bowen and Smith 1976; Davies and Maloney 1976). In the 1980s, Whitehouse and colleagues reported the loss of cholinergic neurons in the nucleus magnocellularis of Meynert in postmortem samples of AD patients (Whitehouse et al. 1982). These studies, along with the proposition of the "cholinergic hypothesis of geriatric memory dysfunction" (Bartus et al. 1982), provoked the formulation of the so-called cholinergic hypothesis of AD. The simplistic view at the time was that there was a correlation between the anterograde loss of dopaminergic neurons in Parkinson's disease and the anterograde loss of cholinergic neurons in AD. Alternatively, our lab proposed (Cuello and Sofroniew 1984) that the cholinergic involvement in AD was

secondary to cortical lesion (retrograde degeneration) based on our experimental evidence for basalis nucleus cell shrinkage following stroke-type cortical lesions (Sofroniew et al. 1983). In more recent years, transgenic animal models of the AD-like amyloid pathology have been able to reproduce this and have also shown an unexpected early increase in the density of cortical cholinergic boutons (Wong et al. 1999; Hu et al. 2003; Bell et al. 2006a, Bell and Cuello 2006).

The causative mechanisms for the vulnerability of forebrain cholinergic neurons in Alzheimer's disease pathology remain unresolved. The basal forebrain cholinergic neurons (BFCNs) projecting from the nucleus basalis and septum provide the bulk of synaptic cholinergic input to the cerebral cortex and the hippocampus, respectively. These neurons have been shown to be highly dependent on NGF supply during adulthood for the maintenance of their biochemical and anatomical phenotype (Thoenen 1995; Cuello 1996). Given this NGF dependence, it was early suspected that the AD pathology brings about a deficit of NGF. However, no consistent evidence was found for the absence of NGF mRNA or for immunoreactive NGF material in the brain of AD sufferers. On the contrary, more recent investigations by Fahnestock and collaborators (Fahnestock et al. 2001) elegantly demonstrated that the NGF precursor molecule, proNGF, is markedly elevated in this neurodegenerative disease. A cholinergic atrophy of NGF-dependent neurons concurrent with elevated supply of the NGF precursor molecule creates an obvious paradox. We believe to have resolved this paradox, firstly through the elucidation of the modality of NGF release and its metabolic pathway in the CNS and, further, through the analysis of this pathway in AD brains.

The NGF Metabolic Pathway in the CNS and its Impact on the Cholinergic Phenotype

For over two decades, it has been assumed that the mature NGF form accounts for the neurotrophin's biological activity, including cell survival, neurite outgrowth, and neuronal differentiation. The realization that proNGF might play a biological role in the CNS (Lee et al. 2001) raised questions regarding the regulatory mechanisms leading to its release, as well as the control of the proNGF-to-mNGF ratio and ultimately the degradation of the mature and biologically active NGF molecule. In order to resolve these outstanding issues, we performed a series of in vitro and in vivo studies. These studies have revealed that proNGF is the main releasable form of the neurotrophin following the stimulation of the cerebral cortex with the transient superfusion of cortical tissue with

neurotransmitters or inducing membrane depolarization with high molarity potassium solutions. We have been able to demonstrate that the conversion of proNGF to mNGF and its degradation occur in the extracellular space with the involvement of a complex protease cascade (Bruno and Cuello 2006). The zymogens, proteases, and endogenous regulators involved in the NGF conversion and degradation following the release of the precursor proNGF are illustrated in Fig. 1.

In brief, we have proposed that the conversion of proNGF to mature NGF is made by the action of plasmin which is itself converted from plasminogen by the action of tissue plasminogen activator (tPA), a mechanism regulated by the protein neuroserpin. The degradation and consequent inactivation of the mature NGF which is not bound to receptors or internalized also takes place in the extracellular space. This process is mediated by the activation of the precursor of matrix metalloproteinase 9 (MMP-9) by plasmin and other agents and its activity regulated by the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1). We have further been able to validate this pathway with in vivo studies. Thus, we have intervened at two levels of the proposed cascade by interfering locally, in the cerebral cortex, with agents capable of inhibiting either the conversion of proNGF to mature NGF or the degradation of NGF. Firstly, we blocked the formation/activation of plasmin by intracortically infusing neuroserpin to inhibit tPA action with an endogenous tPA inhibitor. This procedure provoked in 72 h a several fold increment in the proNGF cortical tissue levels, when compared to the contralateral, vehicle-injected side, as illustrated in Fig. 2a. In contrast, the unilateral infusion of the MMP-9 inhibitor, GM6001, in the cerebral cortex of young rats for 72 h caused a dramatic rise of endogenous mNGF content when compared to values from the contralateral side that received the GM inactive control compound, as illustrated in Fig. 2b.

More recently, we have been able to validate the link between the status of this metabolic pathway and the cholinergic phenotype (Allard et al., in preparation). As illustrated in Fig. 3, we have observed that the prolonged (2 weeks) local inhibition of plasmin activity with the intracortical application of alpha-2-antiplasmin results in the fairly specific diminution of the density of cortical cholinergic presynaptic boutons, as defined by the immunoreactivity of vesicular acetyl choline transporter (VAChT) and measured with a computer-assisted image analysis system. This effect was more noticeable in the sections of the CNS closer to the site of infusion and has little to no effect on the density of the overall (cholinergic and non-cholinergic) presynaptic boutons as revealed by synaptophysin immunoreactivity. Such observations are in line with the in vivo evidence that the steady-state

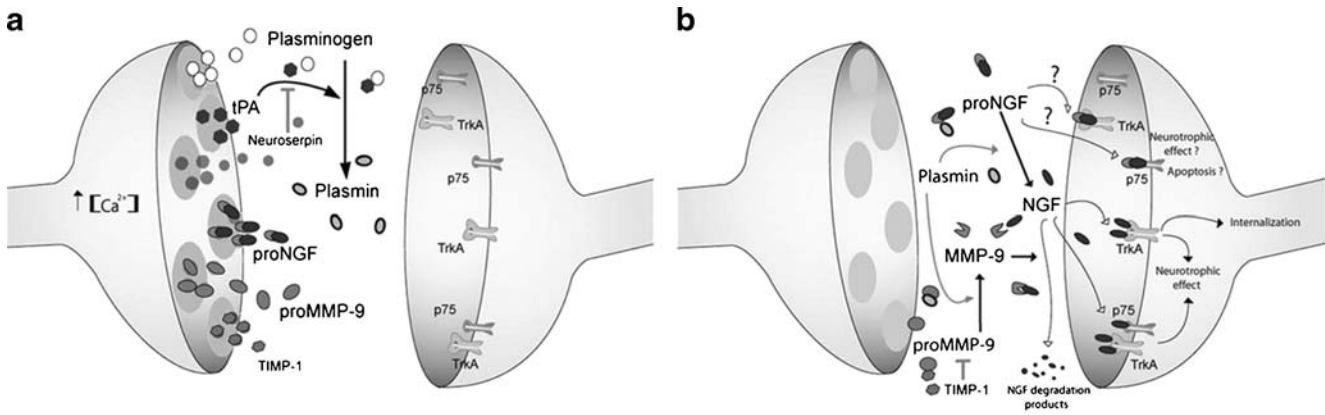**Figure 1** Schematic representation of the events leading to proNGF conversion into mature NGF (**a**) and its subsequent degradation (**b**). Neuronally stored proNGF, plasminogen, tPA, neuroserpin, proMMP-9, and TIMP-1 are coordinately released into the extracellular space upon neuronal stimulation. Released tPA, the activity of which is tightly regulated by neuroserpin, then induces the conversion of plasminogen to plasmin. Plasmin then acts to both convert proNGF into mature NGF and activate proMMP-9 into active MMP-9. Mature NGF in the extracellular space would either then interact with its cognate receptors (TrkA and p75 neurotrophin receptor) or suffer degradation by activated MMP-9. Reproduced with permission from Bruno and Cuello 2006
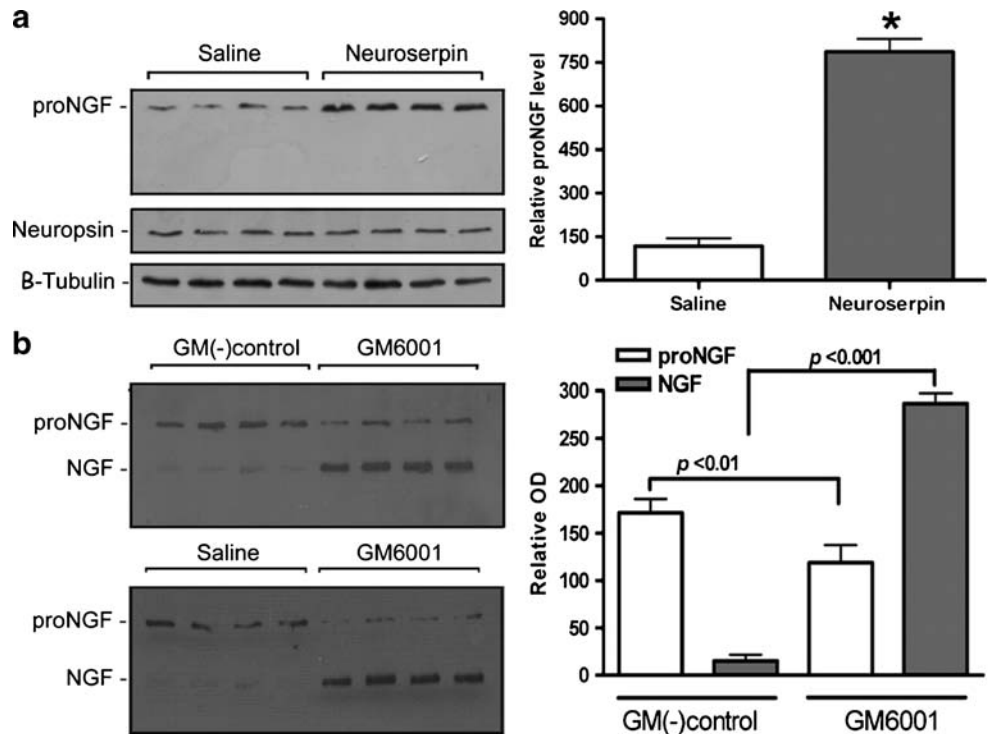
numbers of cortical cholinergic boutons are dependent on the continuous supply of endogenous NGF (Debeir et al. 1999).

Alterations of the NGF Metabolism in Alzheimer's Disease

We have in consequence hypothesized that the levels and activity of the NGF maturation/degradation cascade, rather than the levels of the NGF precursor protein will ultimately define the trophic status of the BFCNs in Alzheimer's disease. In other words, the uninterrupted supply of NGF should maintain the neuronal phenotype of forebrain cholinergic neurons and the failure of this system would provoke a cholinergic atrophy. This has been observed in post-mortem samples of AD patients (Bruno et al. 2006) and is what we have found in the medial frontal cortex of AD. In these investigations, we have observed a failure in the metabolic loop required for

Figure 2 The cortical proNGF–mature NGF ratio is changed by the application of neuroserpin or MMP-9 inhibitors. **a** Increased amount of cortical proNGF in neuroserpin-treated animals (mean±SEM; $P≤0.001$; t test). **b** The inhibitor of matrix metalloproteinase, GM6001, significantly increased the cortical mNGF ($P≤0.001$) and decreased proNGF ($P≤0.01$) when compared with the GM6001 negative or saline control treated (mean±SEM). Reproduced with permission from Bruno and Cuello 2006
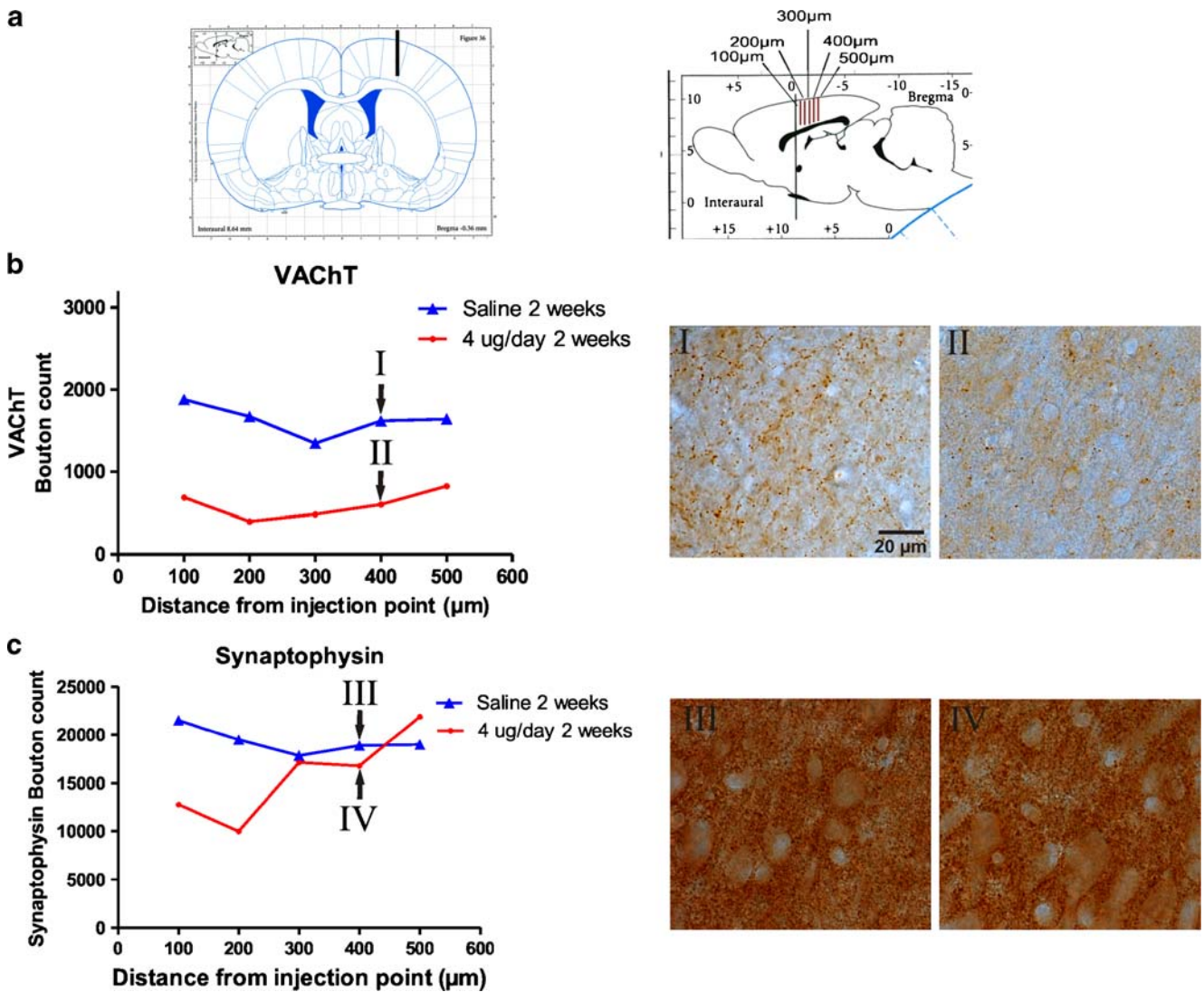
Figure 3 In vivo chronic injection of α_2 antiplasmin affects preferentially cortical cholinergic boutons. **a** Schematic representation of the injection point of either saline or antiplasmin. The cortical cannula had a length of 2.4 mm below pedestal (scull surface) and was inserted at the following coordinates: AP: −0.36; L: 3.0. All coordinates were derived from the Paxinos and Watson rat brain atlas (2005, fifth edition). **b** Schematic representation of the location of the histological sections used for quantification. The sections were taken at a 0.1-mm interval starting at 0.1 mm caudal to the injection point. **c** Quantification of the VAChT immunoreactive (ir) and synaptophysin ir cortical presynaptic boutons. Note that VAChT ir (cholinergic) presynaptic boutons seem more vulnerable to the treatment than the synaptophysin ir (global presynaptic population) as the bouton counts remain lower than in saline-treated animals up to 500 μm caudal to the injection point. Inserts *I–IV* are representative photomicrographs of histological sections taken 400 μm caudal to the injection point

the conversion of proNGF to mNGF. We observed lower levels of the plasmin zymogen (plasminogen) as well as of the corresponding activating protease tPA. The diminished levels of zymogen and tPA results in very low levels of cortical active plasmin (about 25% of control levels) in the AD cerebral cortex, a condition which impairs the efficient "conversion" of proNGF to mNGF and explains the AD apparent paradox of proNGF abundance, concur-

rent with a cholinergic atrophy. This failure of NGF trophic support is further aggravated by the fact that degradation of the mature NGF is exacerbated in AD. We have found that there is an increase in the protein levels and the enzymatic activity of MMP-9, the metalloprotease seemingly responsible for the NGF degradation (Bruno et al. 2006). This leads to a decrease in the CNS supply of endogenous mNGF and has the unavoidable conse-

quence of cholinergic atrophy. Our preliminary studies (Bruno et al. 2008) have shown that the application of Aβ polymers in the hippocampus is sufficient to unleash a similar, AD-like profile of NGF dysmetabolism. Furthermore, such a consequence of the pathological Aβ load is apparently mediated by an inflammatory process as the therapeutic application of minocycline, a well-known CNS anti-inflammatory agent, counteracts both the Aβ-induced inflammation and the NGF dysmetabolism.

In summary, the scenario illustrated in Fig. 4 would create a "feed-forward" pathological cycle in which the progressive accumulation of Aβ will negatively dysregulate the NGF metabolic cascade resulting in cholinergic atrophy. In turn, this cholinergic atrophy will result in diminished production and release of acetylcholine, a transmitter shown to favor a non-amyloidogenic pathway since the activation of M1 and M3 cholinergic receptors stimulates the alpha-secretase type of APP cleavage (Nitsch et al. 1992; Hock et al. 2000). In other words, the shift towards an amyloidogenic metabolism of APP (i.e., higher yields of neurotoxic Aβ peptides) would further, and repetitively, escalate the compromise of the NGF metabolism as schematically represented in Fig. 4.

Conclusions

Our laboratory has shown that endogenous NGF exerts a tight control on the steady-state number of cholinergic synapses in the neocortex. As both pro- and mature NGF forms are present in the CNS, we investigated and elucidated a metabolic pathway responsible for the release, maturation, and degradation of this key neurotrophin. We have validated in vivo the relevance of this pathway. Furthermore, we have observed in Alzheimer's disease brain material a marked dysregulation of the NGF metabolic pathway which can explain the apparent paradox of elevated NGF precursor along with the well-known atrophy of cholinergic neurons found in this neurodegenerative condition. The application of Aβ oligomers was sufficient to reproduce the CNS dysmetabolism of NGF, as observed in AD, suggesting its causality.
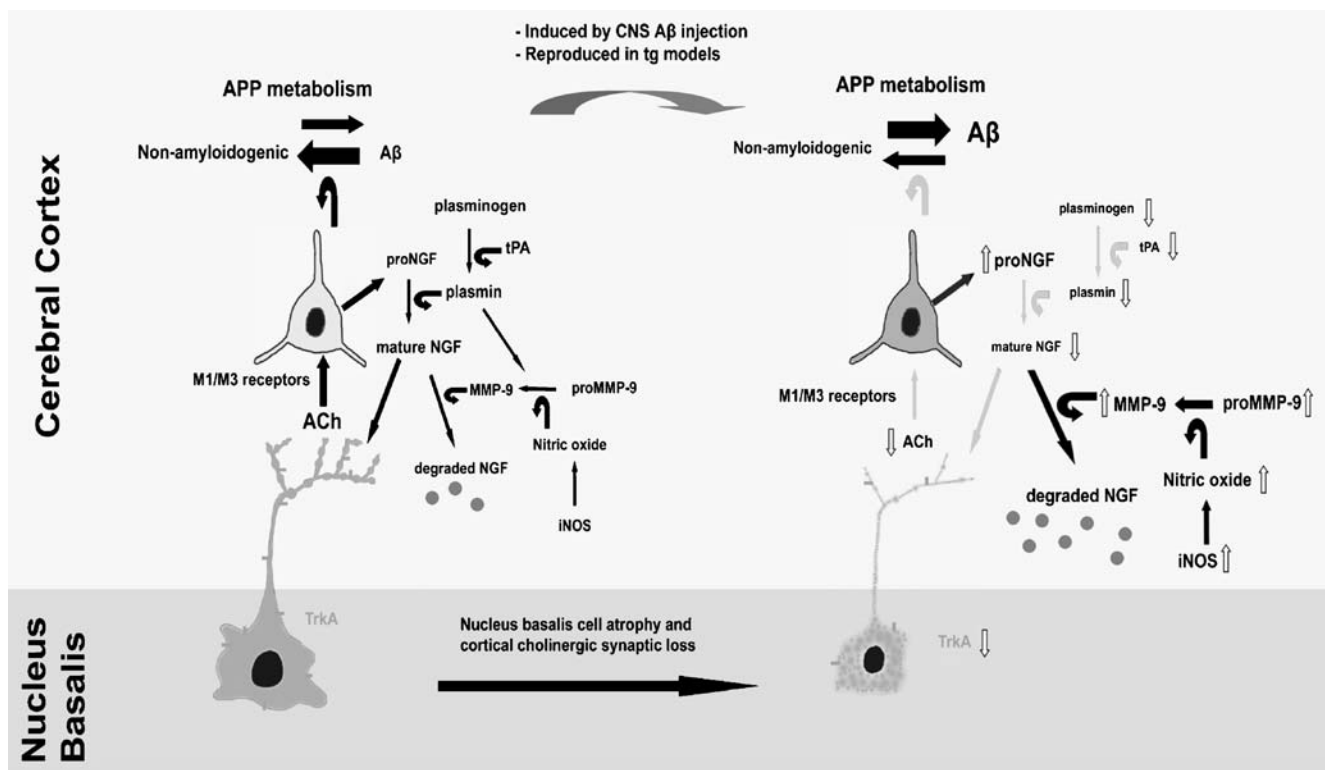
Figure 4 Schematic representation of the effects on NGF metabolism of a shift towards an amyloidogenic metabolism of APP

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