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Targeting NGF-pathway for developing neuroprotective therapies for multiple sclerosis and other neurological diseases

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ABSTRACT

Inflammation is the first line of defense against injury and infection and works both by controlling the ongoing pathological processes and by promoting neuroprotection and regeneration. When the inflammatory response is hyperactivated, it plays a pivotal role in the pathophysiology of many neurological diseases, as it can also be a source of additional injury to host cells. Since neurons lack the ability to divide and recover poorly from injury, they are extremely vulnerable to autodestructive immune and inflammatory processes, and this side effect is fundamental to the outcome of neurological diseases. Inappropriate immune responses are responsible for diseases such as Multiple Sclerosis (MS), Alzheimer's disease (AD) or Parkinson's disease (PD) and for the increased disability after brain trauma or stroke. However, in certain circumstances immune responses in the brain might have a neuroprotective effect, possibly mediated by the release of trophic factors from inflammatory and/or glial cells. The nerve growth factor (NGF) was the first neurotrophin discovered for its stimulatory effect on differentiation, survival, and growth of neurons in peripheral and central nervous system. This factor can protect axons and myelin from inflammatory damage and also can modulate the immune system, reducing the enhanced excitotoxicity during acute inflammatory activation. Therefore, because its neuroprotective activity and immunomodulatory effects, NGF may represent a new therapeutic approach for the treatment of numerous brain disorders.

Key words

Nerve Growth Factor • Multiple Sclerosis • Neuroinflammation • Neuroprotection

Introduction

The neurotrophins are homodimeric polypeptides with pleiotropic activity, able to influence the generation, differentiation, survival, and regeneration of vertebrate neurons (Thoenen, 1991; Persson and Ibanez, 1993). Nerve growth factor (NGF) was the first neurotrophin to be discovered (Cowan et al., 2001) and characterized for its anti-apoptotic role in neuronal development. Other structurally related proteins, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), have similar neurotrophic capacities and, together with NGF, form the neurotrophin protein family (Barde et al., 1983).

The biological activity of NGF is regulated by two different types of receptors expressed by responsive cells: the high-affinity NGF-receptor (TrkA), which belongs to the family of tyrosine kinase receptors, and the low-affinity NGF-receptor (p75), a transmembrane glycoprotein lacking a tyrosine kinase domain (Meakin and Shooter, 1992; Casaccia-Bonnefil et al., 1999).

Neurotrophins act via two primary signalling mechanisms to ensure nervous system cell survival: the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (protein kinase B/v-akt murint thymoma vira oncogene homologue) inhibits the apoptosis-related functions of the forkhead and B-cell leukemia/lymphoma 2 (BCL2)-associated death protein (BAD) in order to

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counteract processes necessary for cell death (Zhang et al., 2000), and the mitogen-activated protein kinase (MAPK)-MEK [MAPK/extracellular signal-regulated kinase (ERK) kinase] pathway which up-regulates via a signalling cascade such anti-apoptotic proteins as BCL2 (Aloyz et al., 1998) and the CREB transcription factor (cyclic AMP responsive element-binding protein) (Riccio et al., 1999).

The neurotrophic factors not only influence the neural development, they also act on mature neurons and particularly on injured and degenerative nerve cells (Lindvall et al., 1994; Tuszynski and Gage, 1995; Lykissas et al., 2007; Song et al., 2009). In a recent study, for example, it was shown that NGF played a protective role on retinal ganglion cell death occurring in glaucoma (Lambiase et al., 2009). Also in the healthy mature nervous system neurotrophins regulate neuronal plasticity, triggering adaptive changes in adult neuronal morphology (McAllister, 2000; Conner et al., 2009; Ohira and Hayashi, 2009), modulating functional properties in presynaptic and postsynaptic mechanisms, and initiating fast synaptic responses (Kafitz et al., 1999; Elmariah et al., 2005). Also, neurotrophins can deeply influence both the synthesis of enzymes involved in neurotransmitter synthesis pathways and the expression of neurotransmitter receptors.

Discovered in 1977 by the work of Aloe and Levi-Montalcini, NGF was the first neurotrophin shown to be synthesized, stored, and released by immune cells (T and B lymphocytes, mast cells, macrophages), that can also respond to NGF in an autocrine manner as they express p75 and TrkA NGF receptors (Ehrhard et al., 1993; Leon et al., 1994; Santambrogio et al., 1994; Villoslada and Genain, 2004). The central nervous system (CNS) is partially isolated from the immune system by the restrictive blood-brain barrier (BBB) and the presence of an immunosuppressive environment (brain immune privilege). Inflammatory attacks in the CNS are controlled by the regulation of local antigen presentation through a special cytokine environment and by the secretion of additional neurotrophic factors (Hickey et al., 1991, 2001; Becher et al., 2006; Kleine and Benes, 2006).

Immune responses can be responsible for diseases such as multiple sclerosis (MS), an inflammatory autoimmune disease in which infiltrating inflammatory immune cells contribute to CNS damage by destroying the myelin sheath of neurons. Also, an inappropriate immune response is responsible for the increased disability after brain trauma or stroke. Inflammation also plays role in neurodegenerative disorders such as Alzheimer's (AD) or Parkinson's disease (PD). It has also been hypothesized that the loss of endogenous target-derived trophic support for selective neuronal populations may lead to the loss of neurons characteristic of AD, PD and other neurodegenerative diseases (Connor and Dragunow, 1998; Drukarch and van Muiswinkel, 2001; Laske et al., 2006).

For these reasons, many studies focused on the development of neuroprotective/immunomodulatory strategies for the treatment of neurodegenerative disorders. A novel approach to restore and maintain the neuronal function in the CNS can be found in the therapeutic application of NGF.

The aim of this review is, therefore, to summarize the evidence for the role of trophic factors in neurodegenerative disorders with a particular interest in the relationship between inflammation, neurodegeneration, and neuroprotection, focusing on the therapeutic use of NGF in human brain diseases.

Neuroinflammation in the pathogenesis of neurodegenerative disorders

Inflammation is the first line of defense against injury and infection, but when the inflammatory response is excessive, it can also be a source of additional injury to host cells (Wiss-Coray and Mucke, 2002). Neurons are extremely vulnerable to autodestructive immune and inflammatory processes as they cannot undergo mitosis and do not recover from injury. For this reason, the CNS is partially isolated from the immune system through the BBB and an immunosuppressive environment. However, it has been shown that in some pathological conditions the privileged immune situation of the brain is altered and immune cells such as activated lymphocytes can cross the BBB (Hickey, 2001). The main component of immunity in the CNS is constituted by the microglia, which represent the monocytes of the brain. Alterations in their microenvironment can induce them to activate rapidly, changing their morphology and acquiring functions that include phagocytosis and secretion of inflammatory mediators (Perry, 2004). Also, astrocytes can contribute to

these processes by releasing local mediators. This localized process is called "neuroinflammation" and it represents a potential pathogenic mechanism in neurodegenerative diseases such as MS, AD or PD. Many neurodegenerative disorders such as AD, PD or ALS are associated with the accumulation of abnormal protein assemblies (Orr and Zoghbi, 2000; Walker and LeVine, 2000; Sherman and Goldberg, 2001) which are able to trigger cellular stress and neuroinflammation. In these circumstances, degenerating cells can provoke inflammation until phagocytes clear them. AD is a age-related disorder that affects people 65 years and older and is the most common dementia associated with progressive neurodegeneration. The brain in AD is characterized by the presence of senile plaques, extracellular deposits of β-amyloid, and neurofibrillary tangles made up of intracellular aggregates of aberrantly phosphorylated Tau protein. Activated microglia surrounding senile plaques, the activation of the complement system, as well as the action of cytokines, chemokines and free radicals have been all associated with AD (McGeer et al., 2001). Also, neuroinflammation drives a self-propagating toxic cycle which induces the increase in β-amyloid deposition and neuronal injury. The neuronal cholinergic degeneration of the *Nucleus basalis of Meynert* (Coyle et al., 1983) leads to the reduction in acetylcholine and cognitive deterioration. Also, it has been proposed that such neurodegeneration is linked to a lack of trophic support. PD is another neurodegenerative disorder characterized by progressive neuronal degeneration. Oxidative stress, excitotoxicity, depletion of endogenous antioxidants, reduced expression of trophic factors and the dysfunction of protein degradation systems are responsible for the cascade of events that leads to neuronal death (Di Monte et al., 1995; Jenner, 2003; Olanow et al., 2003). Also, neuroinflammatory mechanisms may be involved in this pathogenesis.

NGF in brain inflammation

NGF is expressed by immune cells such as T and B lymphocytes, macrophages and mast cells (Ehrhard et al., 1993; Leon et al., 1994; Santambrogio et al., 1994) which in turn have been shown to respond to NGF as they express NGF receptors. The production of this factor is induced by cytokines involved in inflammation and immune responses such as interleukin-1α (IL-1α), interleukin-4 (IL-4), interleukin-5 (IL-5), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and interferon-beta (IFN-β) (Gadient et al., 1990; Awatsuji et al., 1993, 1995; Friedman et al., 1995; Boutros et al., 1997). NGF may be involved in the survival and maintenance of memory B lymphocytes (Torcia et al., 1996), in the stimulation of immunoglobulin production and in the promotion of B-/T-lymphocyte proliferation (Brodie, 1996). NGF can also induce differentiation of monocytes into macrophages and promote their activation, increasing their antimicrobial activity (Table 1).

In MS, the breakdown of the BBB following CNS inflammation allows some immune mediators from the peripheral immune system to cross the barrier. In this condition NGF, which is mainly produced by astrocytes and stored in the extracellular matrix, can prevent the formation of pathogenic inflammatory infiltrates by preventing perivascular mast cells and macrophages from crossing the BBB. This interaction protects CNS integrity and aids in its restoration after inflammation (Flugel et al., 2001). Experimental autoimmune encephalomyelitis (EAE) is one of the most wildly used animal models of MS (Bradl and Linington, 1996). During EAE, both NGF and NGF-receptor expression increase in brain astrocytes, oligodendrocytes and in cells from the subventricular zone (De Simone et al., 1996; Calza et al., 1998; Oderfeld-Nowak et al., 2001). Also, the levels of NGF increase in the cerebrospinal fluid (Laudiero et al., 1992) and in the optic nerve (Micera et al., 1999) of patients affected by MS. This increase may represent a mechanism of response to promote tissue repair and protect CNS tissue against inflammation.

The efficacy of recombinant NGF in promoting myelin repair was evaluated using the EAE model in the common marmoset (Villoslada et al., 2000). This model shows many similarities to MS, including a chronic relapsing course, primary inflammatory demyelination, and changes in magnetic resonance imaging brain scans. This study shows that the continuous intra-cerebroventricular infusion of recombinant human NGF prevents the full development of EAE lesions. The brain of the marmoset treated with NGF showed a decrease in brain inflammation and much less demyelination. This effect

Table 1. - NGF mechanisms of action during brain inflammation.

Target	Effect
Blood-Brain Barrier	Maintenance of Blood-Brain Barrier integrity
Lymphocytes	Switch to the lymphocyte phenotype by avoiding cytotoxicity and inducing immunosuppressive cytokines (IL-10, TGF-β)
Macrophages/microglia	Decrease of antigen presentation by macrophages and microglia by reducing the expression of MHC molecules
Astrocytes	Inactivation of toxic astrocyte mediators
Lymph nodes	Modulation of immune system via sympathetic innervation
Oligodendrocytes	Promotion of myelin maintenance and repair
Neurons	Promotion of axonal survival during inflammation

was attributed to the capacity of NGF to modify the microenvironment in the CNS by inducing an anti-inflammatory effect (down-regulation of IFN-γ production by infiltrating T-cells and up-regulation of IL-10 by glial cells). Indeed, the infusion of NGF led to a reduced breakdown of the BBB. Later studies widened the knowledge base about the regulatory influence of NGF on inflammation. The immunosuppressive property of NGF was confirmed in an EAE-transgenic mouse model carrying the T-cell receptor specific for an encephalitogenic epitope of myelin basic protein (Arredondo et al., 2001). In this model, NGF also modulates the peripheral immune response indirectly through the enhanced sympathetic innervations of lymphoid tissue. In another study, antigen specific Th1 cells designed to deliver NGF in situ ameliorate the course of the inflammatory demyelination of the CNS and peripheral nervous system, inhibiting the migration of immune cells (Kramer et al., 1995; Flugel et al., 2001).

NGF is able to down-regulate the expression of antigen-presenting MHC class II molecules on microglial cells both in culture and in hippocampal slides (Neumann et al., 1998). Furthermore, NGF can influence the expression of the co-stimulatory molecules B7.1 and CD40 by cultured rat microglia (Wei and Jonakait, 1999). Interestingly, the same infiltrating lymphocytes may synthesize not only NGF but also other neurotrophins such as BDNF (Kerschensteiner et al., 1999; Moalem et al., 2000; Muhallab et al., 2002; Stadelmann et al., 2002). Thus, this may represent a self-limiting mechanism of inflammation, that could be malfunctioning during pathological conditions such as MS. It has been shown that infiltrating immune cells can also offer neurotrophic support in experimental models of ischemic, traumatic, or degenerative CNS disorders. Also, activated microglia can synthesize neurotrophins both in culture and in human CNS disease such as human immunodeficiency virus type 1 encephalitis (Elkabes et al., 1996; Soontotnniyomkij et al., 1998). This process differs from neurodegenerative diseases such as PD and AD where, even if microglia and astrocytes are activated, the expression of neurotrophins by resident CNS cells is reduced (Pillips et al., 1991; Mogi et al., 1999).

Overall, several neuropathological conditions may be regulated by neurotrophins (Hefti at al., 1994). Therefore, targeting the neurotrophin mediated signaling pathway provides a rationale for therapeutic intervention. All evidence previously reported shows that neurotrophin therapeutic application could be an interesting way to induce a protective/reparative effect in many neurodegenerative disorders by taking advantages of the pleiotropic proprieties of NGF, which is able to induce both neuroreparative effects in developed CNS and immunomodulation (Fig. 1).

Therapeutic strategies with NGF

Currently the use of disease-modifying immunomodulatory drugs such as the Interferon-β (INF-β) or glatiramer acetate (GA) represent the most used treatment for MS. Although used for the relapsing form of MS, neither INF-β nor GA has demonstrated a significant effect in the progressive disease phase of the disease. Moreover, despite the evidence for efficacy in clinical trials, the individual response among patients to this kind of treatment is heterogeneous (Aktas et al., 2009).

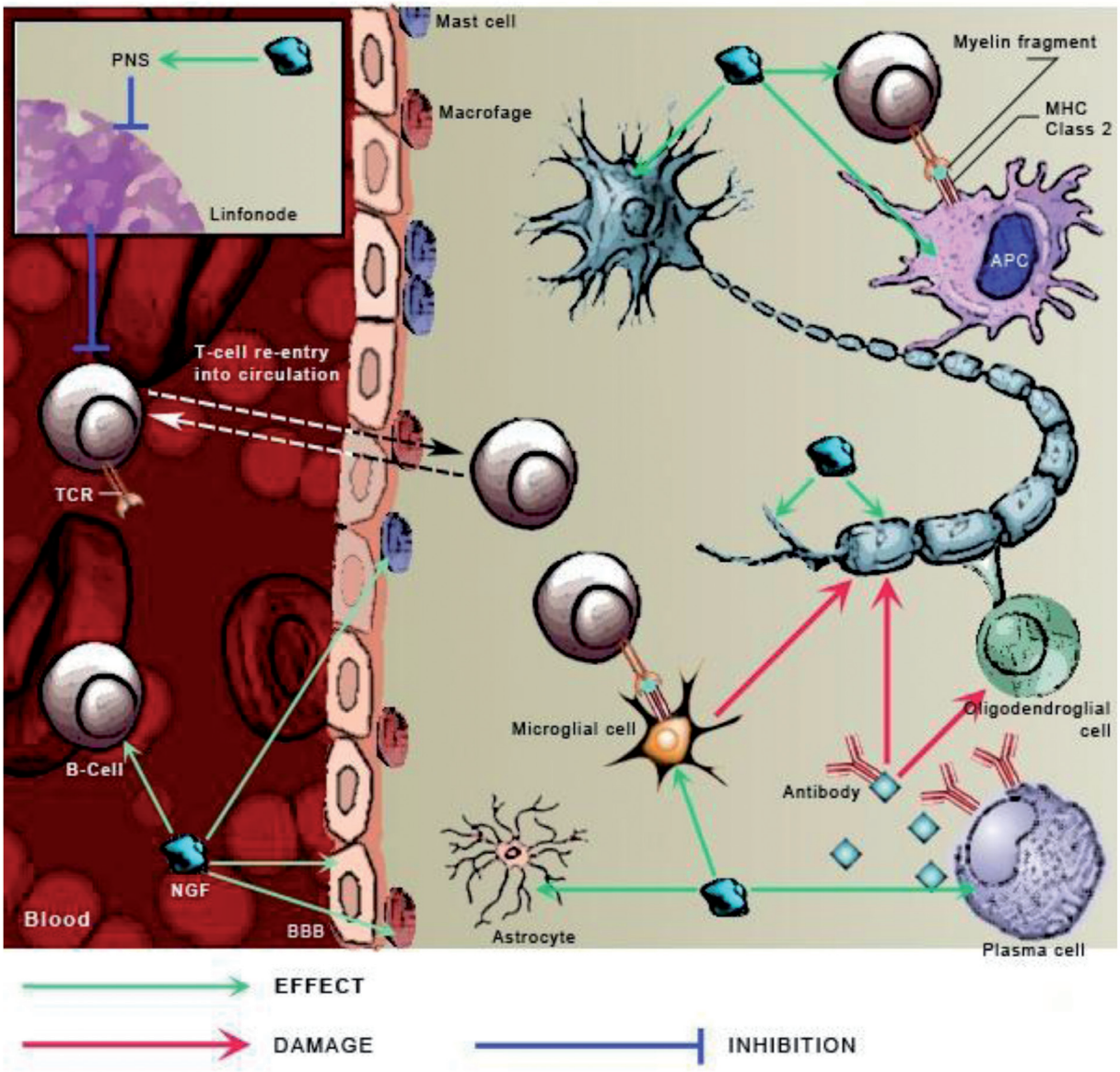

Fig. 1. - Cells and pathways on which NGF may exert a therapeutic effect in neuroinflammation and MS pathogenesis.

Several pharmacological approaches for developing new drugs able to influence neurotrophin function have been attempted. However, despite initial success in animals, clinical trials have been largely disappointing (Saragovi and Burgess, 1999). The use of NGF introduced into the brain as a recombinant protein by intratecal injection might be too invasive for clinical application, while the intravenous/intraperitoneal injection of NGF is not able to reach the CNS and produces secondary effect such as neurological pain (Petty et al., 1994). The failures in the application of the recombinant NGF are not only due to its difficulty crossing the BBB and the undesired pleiotropic effects when applied systemically, but also to the short neurotrophin half-life *in vivo*, poor pharmacokinetics and proteolytic degradation, as well as the high costs for producing this compound (Saragovi and Gehring, 2000).

All of these reasons have opened the way for the study of new approaches for improving neurotrophin delivery and activity. Gene therapy, stem-cell therapy, microencapsulation of neurotrophins, and

microencapsulation of genetically engineered cells that secrete neurotrophins have all been considered as possible ways to deliver NGF into the brain. The *ex vivo* gene therapy has been attempted by implanting into the forebrain genetically modified autologous fibroblasts previously isolated and modified to express human NGF (Tutzynski et al., 2005). After implantation, these cells prevent cholinergic neuronal degeneration after axotomy and neurotoxic lesions in rodents and primates. The *ex vivo* gene therapy was applied in a phase I trial for the therapy of AD (Blesch et al., 2006). In this case autologous fibroblasts were obtained from skin biopsies and were genetically modified to express human NGF by retroviral gene transfer. Cells were injected unilaterally or bilaterally into the *Nucleus basalis* of patients. Cholinergic neurons of the nucleus basalis showed a trophic response to NGF delivery, suggesting that NGF can slow the cognitive decline in AD, even if NGF gene therapy is unlikely to influence amyloid deposition, Tau pathology, or the degeneration of non-cholinergic neurons.

Stem cells, which exhibit a high migratory capacity after brain transplantation, can also be genetically modified to carry new genes. These cells could be used instead of fibroblasts, a cell type also known for their immobility after transplantation, to deliver NGF and prevent the neurodegeneration of the basal forebrain cholinergic neurons (Kim and De Vellis, 2009). Another alternative is *in vivo* gene therapy where vectors can be injected directly, eliminating the need for extensive cell cultivation from individual patients. The AAV virus has become one of the most interesting vectors because it can be produced and purified in large quantity and can transduce non-dividing cells such as neurons, and it also has a prolonged gene expression compared to *ex vivo* gene therapy. This application has also passed to phase I trials for the treatment of AD with no reported adverse events (Blesch et al., 2006).

An additional interesting trial of NGF gene therapy consists in polymer encapsulated cell injection. These cells, once encapsulated, are protected from the host immune response, and therefore xenogenic cells can be used instead of autologous cells from individual patients (Blesch et al., 2006).

Finally the development of small molecules able to exert NGF-like activity is a particularly exciting approach. Two approaches have been used to develop small molecules that either (i) exhibit intrinsic neurotrophic activity that retains receptor specificity, affecting only target cells expressing Trk or p75 receptors, acting as agonists or antagonists of these receptors, or (ii) that boost neurotrophin synthesis. The advantage of the use of these molecules is their relative stability *in vivo*; they remain in circulation for more than 24 hours and have excellent targeting, blood clearance, and bioavailability profiles. These molecules may exert their activity through the direct binding of Trk or p75, contacting the receptors at few key regions, or they may cross the membrane and interfere with the same pathway activated by NGF.

An example of one of these small molecules is the gambogic amide, a selective agonist of the TrkA receptor, which induces tyrosine phosphorylation and activates the downstream signaling pathway involving Akt and MAPKs (Jang et al., 2007). Also, gambogic amide prevents glutamate-induced neuronal cell death and provokes prominent neurite outgrowth in PC12 cells. Gambogic amide specifically interacts with the cytoplasmic juxtamembrane domain of TrkA receptor, triggering its dimerization. Administration of this molecule in mice substantially diminishes kainic acid-triggered neuronal cell death and decreases infarct volume in the transient middle cerebral artery occlusion model of stroke.

Another example of such a small molecule is the Xaliproden, a NGF potentiator and serotonin 5-HT1A receptor agonist that potentiates neurite outgrowth in PC12 cells in the presence of NGF (Padines et al., 1995). Xaliproden also protects motoneuron cultures via activation of the MAPK pathway, and making this molecule another possible candidate for the treatment of neurodegenerative diseases (Price et al., 2007).

Conclusions

The neuroprotective activity of NGF in oligodendrocytes and neurons, together with its immunomodulatory effect, make this protein an attractive candidate for the treatment of CNS inflammatory diseases. Unfortunately, the therapeutic application of recombinant NGF in humans is not possible due to the failure of this protein to cross the BBB. Furthermore, NGF, when systemically administrated, causes severe side effects due to its pleiotropic properties. The difficul-

ties encountered in the clinical application of NGF therapies have opened the way for the study of other possible mechanisms to deliver NGF into the brain and the development of new pharmaceutical formulations that could overcome the limitations imposed by direct NGF application. For this purpose, the development of cell/gene therapy together with the development of new small molecules with agonistic activity represent the new developments for future clinical trials. These studies will hopefully succeed in developing clinically relevant immunomodulatory/neuroprotective strategies that suppress the destructive aspects of inflammation, while simultaneously preserving or even enhancing the beneficial effects of this volatile biological process.

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