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Widespread cortical expression of MANF by AAV serotype 7: Localization and protection against ischemic brain injury

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ABSTRACT

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a secreted protein which reduces endoplasmic reticulum (ER) stress and has neurotrophic effects on dopaminergic neurons. Intracortical delivery of recombinant MANF protein protects tissue from ischemic brain injury *in vivo*. In this study, we examined the protective effect of adeno-associated virus serotype 7 encoding MANF in a rodent model of stroke. An AAV vector containing human MANF cDNA (AAV-MANF) was constructed and verified for expression of MANF protein. AAV-MANF or an AAV control vector was administered into three sites in the cerebral cortex of adult rats. One week after the vector injections, the right middle cerebral artery (MCA) was ligated for 60 min. Behavioral monitoring was conducted using body asymmetry analysis, neurological testing, and locomotor activity. Standard immunohistochemical and western blotting procedures were conducted to study MANF expression. Our data showed that AAV-induced MANF expression is redistributed in neurons and glia in cerebral cortex after ischemia. Pretreatment with AAV-MANF reduced the volume of cerebral infarction and facilitated behavioral recovery in stroke rats. In conclusion, our data suggest that intracortical delivery of AAV-MANF increases MANF protein production and reduces ischemic brain injury. Ischemia also caused redistribution of AAV-mediated MANF protein suggesting an injury-induced release.
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Introduction

The pathophysiological mechanisms of acute stroke involve multiple processes including oxygen and glucose deprivation, excessive glutamate release with corresponding excitoxicity, reactive oxygen species formation, protein and lipid modifications, Ca^{++} dysregulation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress and apoptosis. These processes lead to neurodegeneration in the ischemic area which results in disruption of neural circuits that can affect many behavioral and cognitive functions. Evidence from animal models show that neuronal rewiring and synapse strengthening occurs during recovery from ischemic brain injury (for review see/(Murphy and Corbett, 2009). Thus, three therapeutic strategies for treating ischemic brain injury are: 1) to minimize the acute molecular mechanisms related to neuronal death e.g. endoplasmic reticulum (ER) –stress, mitochondrial dysfunction, excitoxicity and apoptosis; 2) to promote functional enhancement of remaining circuitry; and 3) to increase behavioral and cognitive recovery through "neural rewiring' by promoting new functional neural connections. These latter compensations include neural precursor proliferation and migration, axonal pathfinding, neuritic outgrowth and synaptogenesis. Neurotrophic factors have demonstrated capacity for both of these therapeutic strategies. Viral vectors have been shown to provide sustained neurotrophic factor expression throughout the brain. However, the use of viral vectors encoding neurotrophic factors has not been extensively studied in the stroke literature (for review see/(Lim, et al., 2010).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) was initially characterized as a trophic factor for cultured embryonic dopaminergic neurons (Petrova, et al., 2003). MANF and its homologue, cerebral dopamine neurotrophic factor (CDNF) have been shown to promote survival and recovery of midbrain dopamine neurons (Lindholm, et al., 2007; Petrova, et al., 2003; Voutilainen, et al., 2009). MANF is endogenously expressed in neurons and in non-neuronal tissues (Lindholm, et al., 2008; Mizobuchi, et al., 2007). In the brain, the highest levels of MANF are detected in cerebral cortex, hippocampus and cerebellar Purkinje cells (Lindholm, et al., 2008). MANF expression has been shown to be upregulated by ischemia (Apostolou, et al., 2008; Lindholm, et al., 2008; Tadimalla, et al., 2008; Yu et al., 2010) as well as by endoplasmic reticulum (ER) -stress (Apostolou, et al., 2008; Lee, et al., 2003; Mizobuchi, et al., 2007). We have recently shown that intra- cortical delivery of recombinant MANF protein reduces cerebral infarction and ischemia-mediated

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apoptosis in stroke animals (Airavaara, et al., 2009). Additionally, MANF has been shown to protect cells against glucose deprivation and tunicamycin, a inhibitor of protein glycosylation that induces ER-stress (Apostolou, et al., 2008). Thus, increased MANF levels after injury may be a result of activation of endogenous protective processes against protein misfolding.

Adeno-associated viral (AAV) vectors are currently the predominant viral vector used in clinical trials for neurodegenerative diseases (Lim, et al., 2010). Although the transgene capacity of single (i.e., 4-5 kb) or double (2-3 kb) stranded (McCarty, et al., 2003; Wang, et al., 2003) AAV vectors is limited, the vector genome is amenable to relatively small cDNA sizes which makes them ideal for expressing functional neurotrophic factors such as MANF (<1 kb). The advantage of using dsAAV vectors or self-complementary vectors is higher transduction efficiency and more rapid onset of transgene expression compared to single-stranded AAV vectors (McCarty, et al., 2003; Wang, et al., 2003).

In this study, we generated a serotype 7, dsAAV vector expressing human MANF cDNA for intracortical expression of MANF in a rat model of stroke. We devised a delivery scheme to provide wide spread transduction of cortical cells in the area affected by middle cerebral artery occlusion in rats. AAV-MANF reduced infarction volume and increased post-stroke recovery of locomotor activity and neurological score. Ischemic injury caused a redistribution of MANF in the AAV-MANF treated animals which was consistent with injury-induced secretion of MANF. Overall, our findings show that AAV-mediated delivery of MANF improves the outcome following ischemic brain injury in rats.

Material and Methods

Animals

Adult male Sprague-Dawley rats (250-350 g, Charles River Laboratory) were maintained under a 12-h light–dark cycle. Food and water were freely available in the home cage. Experimental procedures followed the guidelines of the "Principles of Laboratory Care" (National Institutes of Health publication No. 86-23, 1996) and were approved by the NIDA Animal Care and Use Committee.

Primary cortical cell cultures

Neocortical tissue from E15 embryos of timed-pregnant Sprague-Dawley rats were used to prepare neuronal cultures as described previously with modifications (Howard, et al., 2008). Cells (6×10^4 viable cells/well) were plated in 96 well plates coated with poly-D lysine and placed in 37 °C humidified incubator with 5.5% CO2. Cells were fed by 50% media exchange on 4th day in vitro (DIV4) with feed media (plating media without serum or glutamate). Cells were transduced with dsAAV-GFP or dsAAV-MANF on DIV6 using a multiplicity of infection (MOI) of $1\text{-}4\times10^4$. Cells were fed every 2-3 days by 50% media exchange and fixed on DIV13 for immunofluorescent detection of MANF. For hypoxia experiments, plates were placed into to hypoxia chambers (Billups-Rothenberg Inc., Del Mar, CA, USA) on DIV12 and flushed with nitrogen gas for 1 minute then sealed. Hypoxia chambers were then placed into humidified incubators for 8 hours at 37 °C. Plates were removed from hypoxia chambers and cells were allowed to reoxygenate for 24 hours. Cells were fixed by treating with 4% PFA for 1 hour then returned to PBS for immunostaining.

Construction, Packaging, Purification, and Titering of AAV Vectors

Viral construction, packaging and characterization: The cDNA for eGFP was removed from dsAAV-GFP (Wang, et al., 2003) using the restriction enzymes KpnI and XbaI and replaced with the human MANF cDNA using KpnI and XbaI digestion of PCR3.1-MANF (Lindholm, et al., 2008) to create pdsAAV-MANF. The plasmid was sequence verified and transfected into HEK293 cells. Twenty-four hours after transfection, cell lysates were made and analyzed by western blot using a rabbit anti-human MANF antibody (Lindholm, et al., 2008). Viral stocks of AAV-MANF were prepared using the triple-transfection method (Howard, et al., 2008; Xiao, et al., 1998). Briefly, twenty 15 cm dishes containing HEK293 cells at 85-95% confluency were transfected by the CaCl2 method with pHelper (Stratagene, La Jolla, CA), pdsAAV-GFP or pdsAAV-MANF and a plasmid containing rep/cap genes for serotype7, pAAV7 (Gao, et al., 2002). Plasmids used for packaging AAV were generously provided by Dr. Xiao Xiao (UNC, Chapel Hill, NC). Approximately 48 hours post-transfection, cells were harvested, lysed by freeze/thaw, and purified by centrifugation on a CsCl gradient. Final samples were dialyzed in PBS containing 12.5 mM $MgCl_2$ to $\sim10^{13}$vg/ml, aliquoted and stored at -80 °C until use. All vectors were titered by quantitative PCR using the CMV promoter as the target sequence. Viral titers are recorded as viral genome/ml.

Intracerebral AAV injections

Animals were anesthetized with chloral hydrate (0.4 g/kg, i.p.). AAV-MANF was given intracerebrally into three cortical sites in the distribution of the middle cerebral artery (Fig. 1). The stereotaxic coordinates were AP +1.2 (site 1), -0.3 (site 2), -1.8 (site 3); ML +5.5; DV -3.5 from the skull. Two microliters of AAV-MANF or AAV-GFP (serotype 7, titer $\sim10^{13}$ vg/mL) were injected using a 10 ul Hamilton syringe with a 30 G blunt needle and the needle was lifted by 2 mm at the middle of the injection. The rate of infusion (0.5 μl/min per site) was controlled using a microprocessor controlled injector mounted to a stereotaxic frame (UMP4; World Precision Instruments, Sarasota, FL, USA). The needle was slowly removed 2 minutes after completion of each injection.

Immunofluorescent staining

Seven days after viral injections, rats were perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were stored in 18% sucrose and sectioned coronally or sagittally (40 μm) using a Leica cryostat. Sections were rinsed three times with phosphate buffer (PB) for 10 min and incubated for 1 h with blocking solution (4% BSA in 0.3% Triton X-100 in PB). Sections were incubated overnight with a rabbit anti-MANF antibody (1:100, / (Lindholm, et al., 2008)) and mouse anti-GFAP (glial fibrillary acidic protein, 1:300, Chemicon, Temecula, CA, USA) or mouse anti-NeuN (neuronal nuclei, 1:200, Chemicon). The sections were rinsed three times in PBS for 10 min. The bound primary antibody was visualized using the AlexaFluor 488 goat anti-rabbit or AlexaFluor 568 goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA). Sections were rinsed three times with PB and incubated with DAPI (4,6-diamidino-2-phenylindole, 1:2000; Invitrogen) for 15 min. Brain sections were examined with a Nikon eclipse 80i microscope with Q-imaging camera or with Nikon D-eclipse C1 confocal imaging system equipped for epifluorescence.

In vitro immunofluorescent staining of rat primary cortical cultures was conducted as described previously (Howard, et al., 2008). Briefly, cells were permeabilized for 15 min then blocked for 1 hour with PBS containing 0.1% Triton X-100, 2% BSA, and 5% serum. Cells were incubated over night with rabbit anti-MANF antibody (Lindholm, et al., 2008) , 1:250, in PBS containing 0.1% Triton X-100 and 5% goat serum at 4 °C with gentle shaking. Cells were washed 3 times with PBS for 3 minutes and then incubated for 1 h with AlexaFluor 568 goat anti-rabbit secondary antibody (Invitrogen) and washed again with PBS. Immunofluorescence was visualized and imaged using Nikon