

DISEASES AND DISORDERS

Poststroke delivery of MANF promotes functional recovery in rats

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Stroke is the most common cause of adult disability in developed countries, largely because spontaneous recovery is often incomplete, and no pharmacological means to hasten the recovery exist. It was recently shown that mesencephalic astrocyte-derived neurotrophic factor (MANF) induces alternative or M2 activation of immune cells after retinal damage in both fruit fly and mouse and mediates retinal repair. Therefore, we set out to study whether poststroke MANF administration would enhance brain tissue repair and affect behavioral recovery of rats after cerebral ischemic injury. We used the distal middle cerebral artery occlusion (dMCAo) model of ischemia-reperfusion injury and administered MANF either as a recombinant protein or via adeno-associated viral (AAV) vector. We discovered that, when MANF was administered to the peri-infarct region 2 or 3 days after stroke, it promoted functional recovery of the animals without affecting the lesion volume. Further, AAV7-MANF treatment transiently increased the number of phagocytic macrophages in the subcortical peri-infarct regions. In addition, the analysis of knockout mice revealed the neuroprotective effects of endogenous MANF against ischemic injury, although endogenous MANF had no effect on immune cell-related gene expression. The beneficial effect of MANF treatment on the reversal of stroke-induced behavioral deficits implies that MANF-based therapies could be used for the repair of brain tissue after stroke.

INTRODUCTION

Every year, nearly 800,000 Americans suffer from stroke. Of these, about 660,000 people survive, but the functional recovery is often incomplete. The only drug available for acute treatment of stroke is tissue plasminogen activator, but the short time window for treatment efficacy and a number of contraindications make this a feasible treatment option for only a minority of patients (1). This inability to limit brain damage in the acute phase of stroke and the infrequency of complete spontaneous functional recovery make stroke the leading cause of long-term disability (2, 3). Moreover, the only known effective ways of enhancing recovery—cognitive therapy and physiotherapy—have only a limited effect (4), and there are no pharmacological treatments available to enhance the natural recovery process in patients.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a protein with neuroprotective and neurorestorative effects. We have recently shown that MANF mediates its neuroprotection both in a cell autonomous manner and when applied extracellularly (5). Furthermore, our studies suggest that exogenous MANF is neuroprotective against stroke, but the role of endogenous MANF in ischemic injury is not known (6–8). In a recent study by Neves and colleagues (9), MANF was found to recruit innate immune system cells after retinal injury, cause them to up-regulate the expression of immune cell markers associated with alternative or M2-type activation, and enhance the integration of photoreceptors transplanted into the mouse retina. The proposed role of MANF in central nervous system (CNS) tissue repair could also be important after stroke, where the inflammatory response is massive and contributes to the speed of recovery (10).

Here, we aimed to find out whether a late postischemic delivery of MANF after focal cerebral ischemia hastens functional recovery and influences the processes of tissue repair in the peri-infarct region. As documented in four independent experiments, we found that MANF, delivered to the peri-infarct area via an adeno-associated virus (AAV) vector or as recombinant protein 2 to 3 days after transient focal ischemia, enhanced the behavioral recovery. Our results suggest that MANF transiently increases the number of phagocytic innate immune cells but without selectively recruiting alternatively activated macrophages.

RESULTS

Overexpression of hMANF in the peri-infarct area enhances the reversal of behavioral deficits caused by ischemic injury

We decided to evaluate whether MANF could promote functional recovery when delivered after stroke and took advantage of the recently developed approach for poststroke targeting of genes to the peri-infarct region (5). The method uses the injection of AAV7-MANF on day 2 after the distal middle cerebral artery occlusion (dMCAo) surgery, thereby bypassing the possibly confounding influence its neuroprotective effect has on recovery because, by that time, most of the ischemia-induced cell death has already occurred (Fig. 1A) (11–13). At day 14 after the dMCAo surgery, human MANF (hMANF) and green fluorescent protein (GFP), used as a control, were found to be expressed in the peri-infarct region (Fig. 1, B and C, and fig. S1) (5).

The effect of AAV7-MANF injection on behavioral recovery of the animals was evaluated on days 7 and 14 after the stroke surgery. Compared to the control group of AAV7-GFP-injected animals, AAV7-MANF-injected rats had significantly milder neurological deficits at both time points when assessed with Bederson's neurological score test (BNST) and the elevated body swing test (EBST) (Fig. 1, D and E). By day 14, the AAV7-MANF-injected rats did not exhibit forepaw use bias in the cylinder test, although the difference between the groups did not reach statistical significance (Fig. 1F).

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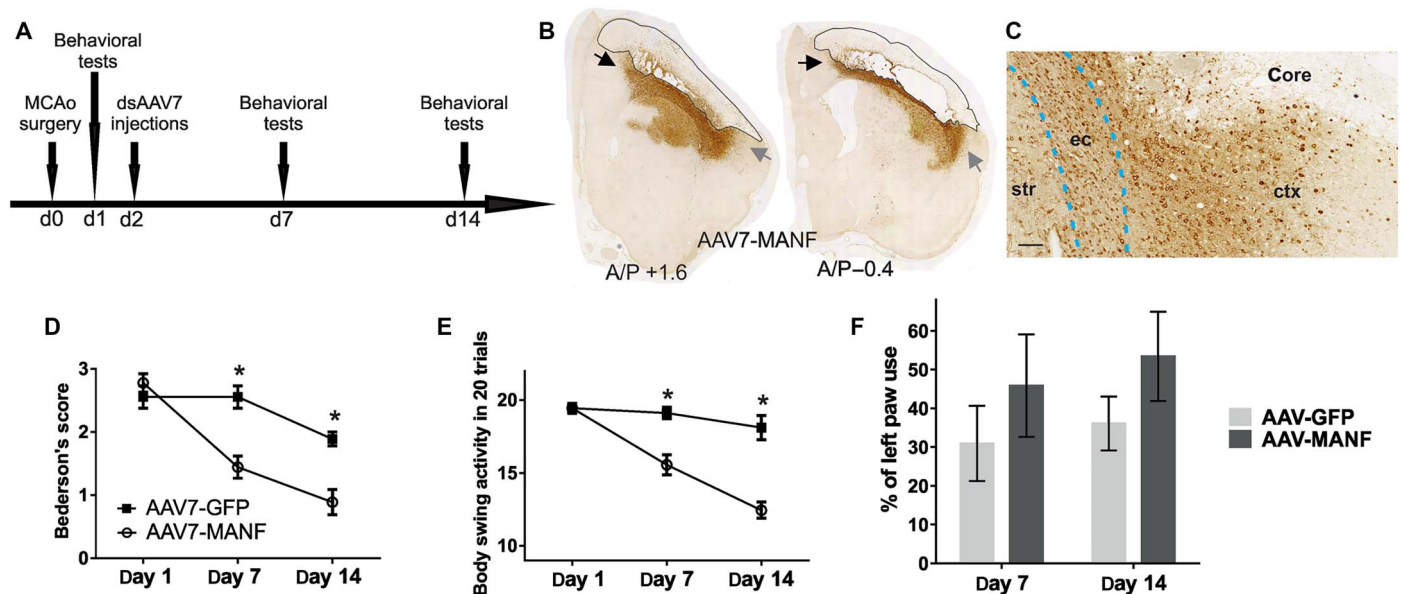


Fig. 1. AAV7-MANF injection after ischemia results in enhanced behavioral recovery. (A) The rats underwent dMCAo surgery, and on day 1, they were divided into two groups, balanced according to the severity of their neurological deficit assessed by the EBST and BNST. On day 2, the animals received intracerebral injections of either AAV7-MANF or AAV7-GFP, and their behavioral recovery was evaluated on days 7 and 14. (B) Expression patterns of hMANF 2 weeks after intracerebral AAV7-MANF injection. Coronal sections of the lesioned cerebral hemisphere were immunostained using anti-hMANF antibody. Sections from positions close to injection sites (at approximately A/P +1.6 and A/P -0.4) are presented. The core of the ischemic lesion is outlined. Black and gray arrows mark the dorsal and lateral peri-infarct cortex, respectively, where the deeper layers are AAV7-transduced. (C) Close-up of hMANF expression in the peri-infarct region encompassing cortex (ctx), striatum (str), and external capsule (ec). Scale bar, 100 μ m. (D and E) The behavior of rats was assessed with BNST (D) and the EBST (E) on days 7 and 14 after the dMCAo surgery ($n = 9$ in both groups). $*P < 0.003$ by Mann-Whitney U test. (F) Forepaw use bias of AAV7-GFP- and AAV7-hMANF-injected rats as determined using the cylinder test. According to a two-way analysis of variance (ANOVA) [$F_{(1,32)} = 2.2056$, $P = 0.1613$], the differences in right forepaw use were not statistically significant. Average \pm SEM is shown.

Spontaneous motor activity did not differ between the treatment groups (fig. S2).

The beneficial effect of MANF on resolving ischemic injury-induced behavioral deficits was also seen when recombinant hMANF (rhMANF) was delivered chronically into the cortex of the lesioned hemisphere over 2 weeks, starting at day 3 after dMCAo surgery (Fig. 2A). Compared to the control group of vehicle-infused rats, rats infused with rhMANF exhibited faster reversal of injury-induced behavioral deficits in the EBST and cylinder tests (Fig. 2, B and C). According to the BNST, the difference between groups was not significant (Fig. 2D).

Next, we investigated whether poststroke MANF limits the size of the developing lesion. We used magnetic resonance imaging (MRI) to follow changes in lesion size within subjects during the recovery period (Fig. 3A). In addition, to exclude possible detrimental effects of the AAV7-GFP virus, we added a control group of rats injected with phosphate-buffered saline (PBS) solution. Similar to the previous experiment, rats that had received intracerebral injection of AAV7-MANF showed less severe stroke-induced behavioral deficits on day 16 compared to both control groups (Fig. 3, B and C). Next, the volume of ischemic lesion was determined from T2-weighted images of MRI performed on day 2, right before the AAV-vector injections, and on days 9 and 16 after the dMCAo surgery (Fig. 3, D and E, and fig. S3, A and B). The change of lesion volume during the recovery period was not different between the treatment groups (Fig. 3, F and G), a conclusion confirmed by histological analysis (fig. S3, C and D). Neither did postischemic MANF overexpression affect the delayed loss of neurons in the area bordering the infarct (fig. S3E) (14). Together, the expression of MANF during the postischemic period can enhance functional recovery without reducing the size of the ischemic lesion.

AAV7-MANF increases the number of phagocytic immune cells after cerebral ischemic injury

We investigated the possible involvement of different processes of damage and tissue repair, which have previously been shown to correlate with behavioral recovery. According to immunohistochemical analysis of rats sacrificed 2 weeks after dMCAo, there were no treatment-induced changes in blood vessel density/angiogenesis (fig. S4, A to D), density of synapses/synaptogenesis (fig. S4, E and F), integrity of white matter (fig. S5), and astrogliosis (fig. S6) in the peri-infarct area.

Next, we took the unbiased approach of looking at possible AAV7-MANF-induced changes in gene expression in the lateral peri-infarct cortex. We performed transcriptome analysis of peri-infarct cortex samples collected on day 4 after the ischemia/reperfusion surgery, that is, 48 hours after the intracerebral administration of either AAV7-GFP or AAV7-MANF. By that time, hMANF and GFP were already well expressed in the peri-infarct area (fig. S7A), and the effect of AAV7-MANF could be seen as a reduction of the behavioral deficit in both the EBST and BNST (fig. S7, B and C). The top hits among genes differentially expressed in the peri-infarct cortex of AAV7-GFP-treated versus AAV7-MANF-treated rats were related to immune cell function (table S1). Quantitative polymerase chain reaction (qPCR) analysis of these genes verified that, after AAV7-MANF treatment, the expression of both *Emr1* (F4/80) and complement component 3 (*C3*) was up-regulated approximately threefold, whereas the increase in complement factor properdin (*Cfp*) expression was statistically insignificant (Fig. 4A). The differential expression of genes associated with phagocytic immune cells implies that poststroke delivery of MANF regulates the activity or number of these cells after ischemic injury. Notably, AAV-MANF treatment did not increase the expression of *Cd8a*, a gene expressed

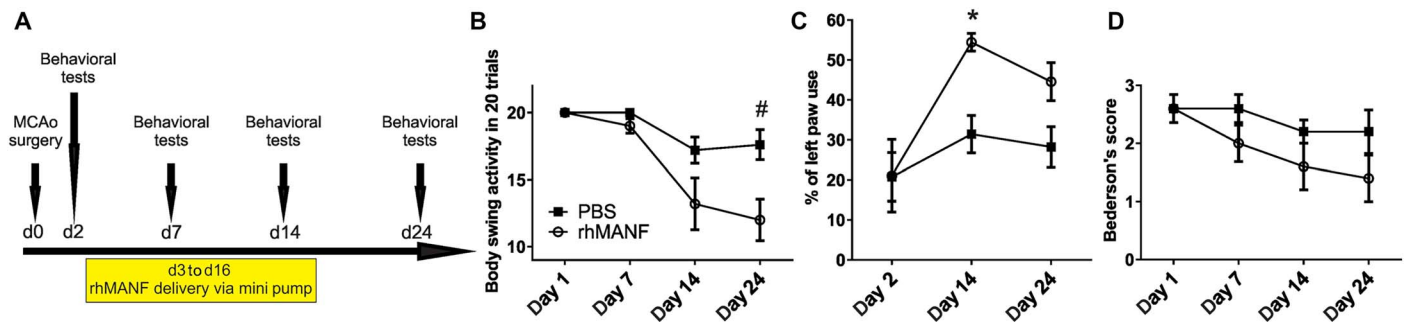


Fig. 2. Chronic delivery of rhMANF results in enhanced behavioral recovery. (A) The rats underwent dMCAo surgery, and on day 2, they were divided into two groups, balanced according to the severity of their neurological deficit assessed by BNST. Intracerebral infusion of PBS ($n = 5$) or rhMANF ($n = 5$) was started 1 day later, with the cannula implanted into the peri-infarct region. On days 7, 14, and 24 after the dMCAo surgery, the behavior of rats was assessed with the EBST (B) and BNST (D). $^{\#}P = 0.0317$ by Mann-Whitney U test. (C) Forepaw use bias of the rats was assessed in the cylinder test on days 2, 14, and 24 after dMCAo. $^*P = 0.0257$ by Bonferroni's multiple comparisons test, following two-way ANOVA [effect of treatment: $F_{(1,24)} = 8.103$, $P = 0.0089$]. Average \pm SEM is shown.

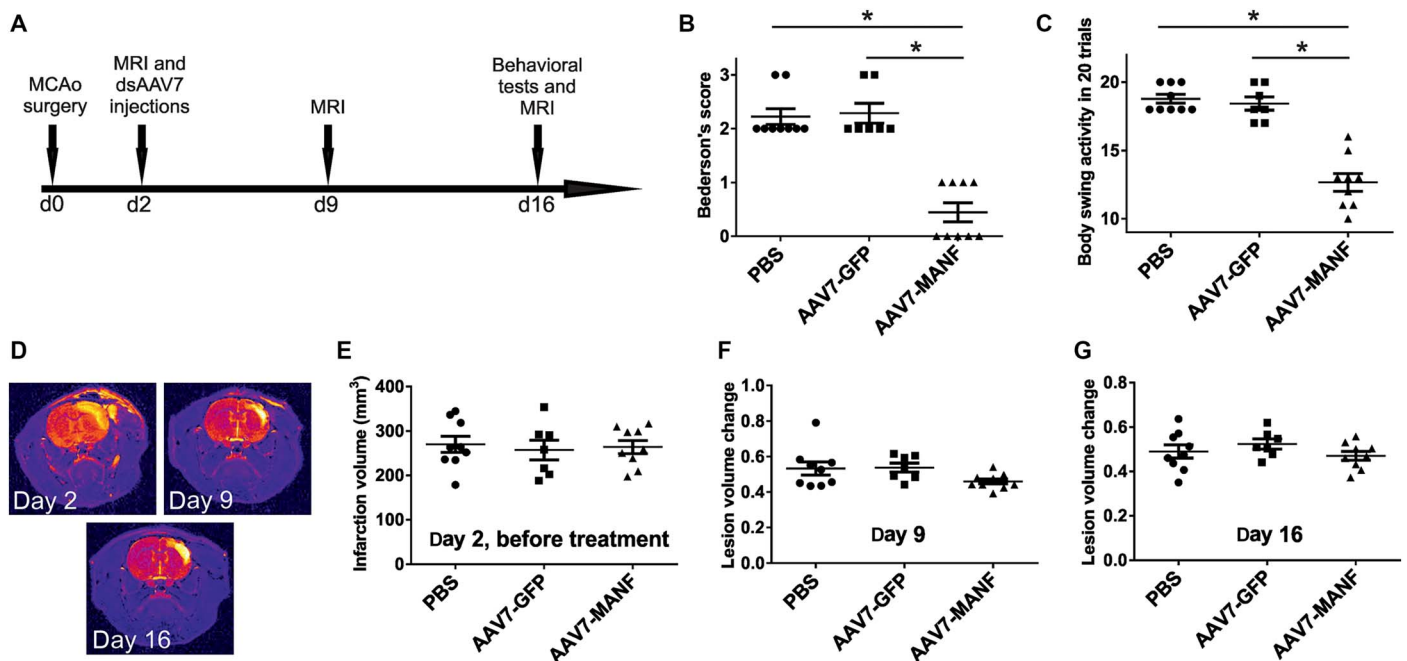


Fig. 3. AAV7-MANF injection after ischemia results in enhanced reversal of behavioral deficits but does not reduce lesion size. (A) The rats underwent dMCAo surgery, and on day 2, they were divided into three groups, balanced according to size of ischemic lesion, as determined from T2-weighted MRI images. On the same day, rats received intracerebral injections of PBS ($n = 9$), AAV7-GFP ($n = 7$), or AAV7-hMANF ($n = 9$). The severity of their neurological deficit was assessed on day 16 using BNST (B) and the EBST (C). $^*P \leq 0.0043$ in Dunn's multiple comparisons post hoc test, following Kruskal-Wallis test ($P \leq 0.0002$). (D) T2-weighted MRI images representative of the development of lesion size from days 2 to 16 after dMCAo surgery. (E to G) The volume of ischemic lesion was measured from T2-weighted MRI images taken on days 2, 9, and 16 after the dMCAo surgery. For days 9 (E) and 16 (G), the lesion volume is expressed in relation to the size of the lesion in the same animal on day 2. $P \geq 0.1079$ according to one-way ANOVA. Average \pm SEM is shown.

specifically in cytotoxic T lymphocytes (Fig. 4A), arguing against the possibility that AAV7-MANF treatment led to an unspecific increase in immune cell infiltration into the peri-infarct cortex. Immunohistochemical analysis revealed that at 48 hours after AAV7-MANF administration, there were more CD68-expressing phagocytic cells in the subcortical white matter (external capsule), where AAV7-MANF is highly expressed, and in the dorsal striatum (Fig. 4, B to D), but not in the peri-infarct cortex (Fig. 4, E and F). There was no significant difference in the infarct area size on the sections analyzed (fig. S8A). In the subcortical white matter, the number of myelin basic protein (MBP)-positive myelin fragments colocalizing with CD68 was also

increased, indicating that, after AAV7-MANF injection, there is increased phagocytosis of myelin debris (fig. S8, B and C). This increase was likely due to the greater number of CD68-expressing cells because there was no increase in the average number of MBP-positive objects per phagocyte (fig. S8D). Notably, at 2 weeks after AAV injection, the number of CD68-expressing phagocytic cells in the peri-infarct region did not differ between the treatment groups (fig. S9, A to E).

Exogenous MANF has been proposed to recruit innate immune cells after retinal damage, possibly directing them toward alternative activation (9). Although AAV7-MANF delivery also transiently increased the number of cells expressing a marker of alternative activation, arginase-1 (Arg1)

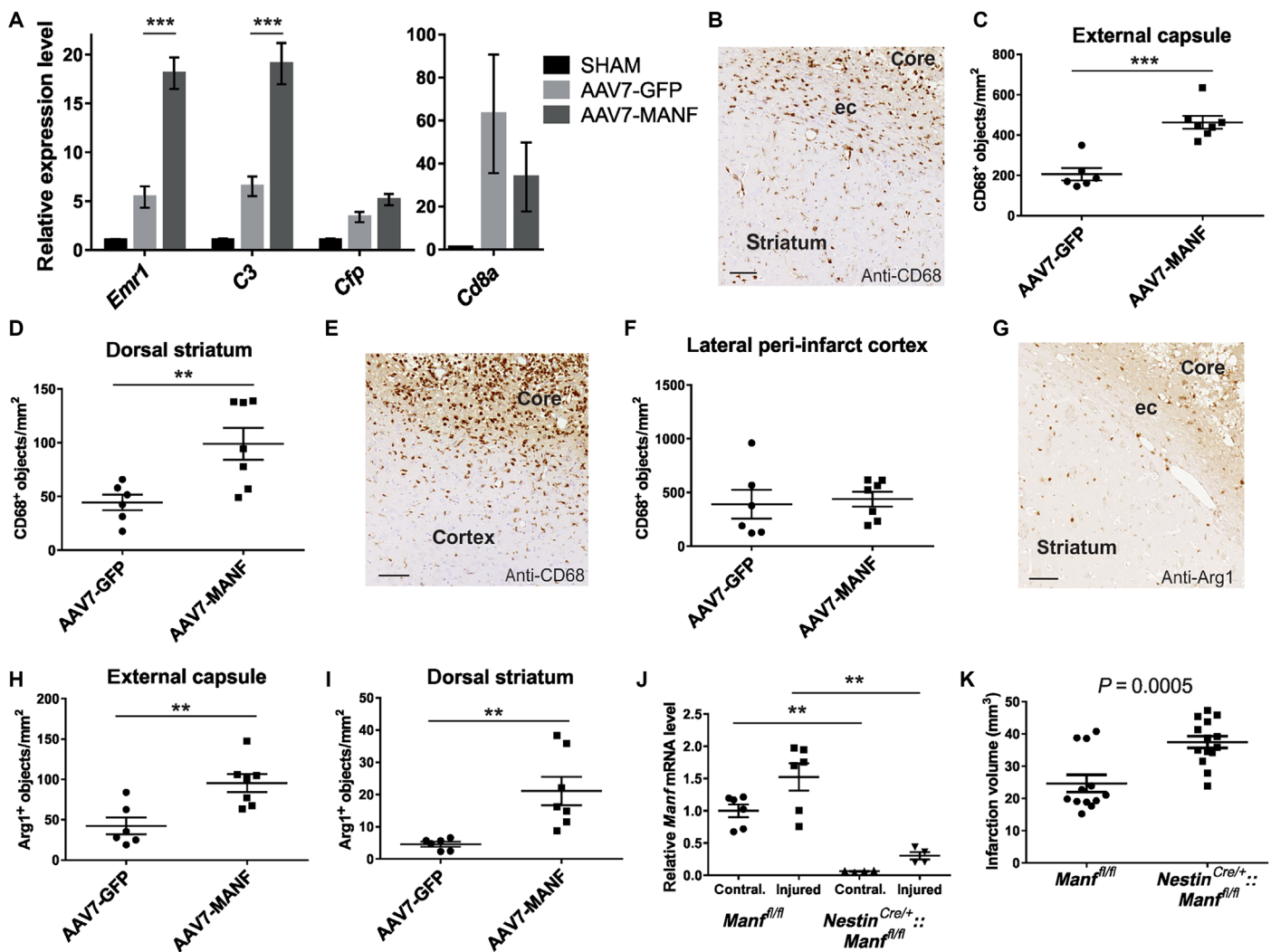


Fig. 4. AAV7-MANF treatment increases the number of phagocytic immune cells in the subcortical regions. (A) qPCR analysis of *Emr1*, *C3*, *Cfp*, and *Cd8a* mRNA levels in the peri-infarct cortex at day 4 after ischemic injury ($n = 4$ to 5). *** $P < 0.0004$ by Tukey's multiple comparisons post hoc test, following one-way ANOVA ($P < 0.0001$). (B and C) Density of CD68⁺ objects in the corpus callosum/external capsule underlying the infarct at day 4 after ischemic injury. *** $P = 0.0001$. (D) Density of CD68⁺ objects in the dorsal striatum. ** $P = 0.0099$. (E and F) Density of CD68⁺ objects in the lateral peri-infarct cortex. $P = 0.7504$. (G and H) Density of Arg1⁺ objects in the corpus callosum/external capsule. ** $P = 0.0053$. (I) Density of Arg1⁺ objects in the dorsal striatum. ** $P = 0.006$. (C to I) The individual points on graphs present the average of two coronal sections, taken from positions A/P +0.1 and -0.7. AAV7-GFP ($n = 6$) or AAV7-hMANF ($n = 7$). Representative image is shown for each area. Scale bars, 100 μm . (J) qPCR analysis of *Manf* mRNA levels in the injured and contralateral (control) cortex of *Manf*^{fl/fl} and *Nestin*^{Cre/+::Manf}^{fl/fl} mice at 2 days after focal ischemic injury ($n = 4$ to 6). ** $P < 0.002$ by Tukey's multiple comparisons post hoc test, following one-way ANOVA ($P < 0.0001$). (K) Infarction volumes (averaged with SEM) in *Manf*^{fl/fl} and *Nestin*^{Cre/+::Manf}^{fl/fl} mice sacrificed 2 days after a permanent MCAO. Lesion volumes were quantified from triphenyl tetrazolium chloride-stained brain sections. Student's *t* test was used for the analysis of statistical significance. Average \pm SEM is shown.

(Fig. 4, G to I, and fig. S9, F to J), their number was much lower compared to the number of CD68-expressing cells. This indicates that the effect of AAV7-MANF on the activation or recruitment of phagocytic immune cells was apparently not specific to alternatively activated immune cells. In addition, AAV7-MANF treatment did not cause any collective down- or up-regulation of M2-type marker genes in the peri-infarct cortex, indicating that there was no MANF-induced shift toward M2-type polarization of the immune cells present in this region (fig. S10).

In the CNS, the expression of *Manf* is ubiquitous, and ischemic injury up-regulates its expression in both neurons and glia (15, 16). We observed that the genetic deletion of *Manf* in cells of neural origin had a

great effect on the overall levels of its transcript in both injured and contralateral cerebral cortex (Fig. 4J). The volume of ischemic infarct was significantly larger in the mice lacking MANF in the nervous system (Fig. 4K), showing that endogenous MANF has a neuroprotective role. We tested whether the up-regulation of MANF in cells of neural origin would affect immune cell-related gene expression after cerebral ischemic injury. Contrary to the effect of AAV7-MANF on gene expression in the peri-infarct cortex, the injury-induced up-regulation of *Emr1* and *C3* in the cerebral cortex was not different between *Nestin*^{Cre/+::MANF}^{fl/fl} and *MANF*^{fl/fl} control mice (fig. S11, A and B). In addition, in contrast to the proposed function of MANF in biasing immune cells toward alternative activation (9), genetic reduction of endogenous

MANF levels did not affect the induction of M2-type activation marker genes *Arg1* and *Mrc1* after ischemic injury (fig. S11C).

DISCUSSION

Here, we have presented data showing that poststroke delivery of hMANF accelerates the reversal of behavioral deficits in rats with cerebral ischemic injury. We report that hMANF transiently increases the number of phagocytic macrophages in the subcortical region. In addition, we show that endogenous MANF is protective against cerebral ischemic injury.

We previously demonstrated that hMANF has the ability to influence the pathophysiological mechanisms of ischemic cell death, being neuroprotective in the rat model of cerebral ischemia when administered before the MCAo surgery (6, 7). Here, we report for the first time that endogenous MANF, expressed in Nestin-positive cells, also protects brain tissue from ischemic injury. We show that in addition to its neuroprotective effect, MANF can speed up the reversal of injury-induced behavioral deficits in rats even when administered days after transient cerebral ischemia. The effect of MANF on recovery was seen upon AAV-mediated delivery and also after chronic infusion of recombinant MANF. Consistent with the full development of lesion within the first days after ischemia (11–13), our MRI results and histological analyses indicate that poststroke delivery of MANF does not affect neuronal cell death.

In contrast to other studies that have reported delayed behavioral improvements after poststroke viral vector-mediated delivery (17, 18), the effect of AAV7-MANF treatment was seen already on days 2 and 5 after the AAV vector delivery. The fact that we saw robust hMANF and GFP expression already at 48 hours after double-stranded AAV vector injections with both immunohistochemistry (fig. S7A) and transcriptome analysis (table S1) agrees with what is known about the time course of expression of self-complementary AAV vectors in brain tissue (19). The promptness of AAV7-MANF's effect on behavior possibly points to a mechanism rather different from the tissue-repair processes usually correlated with behavioral recovery in experimental stroke studies. Histological analysis of blood vessel density, astrogliosis, synaptic protein expression, and damage to white matter at 2 weeks after AAV7-MANF delivery failed to identify histological changes that would correlate with the behavioral effect of AAV7-MANF treatment. However, analysis of MANF-induced changes to gene expression in the peri-infarct cortex at an earlier time point revealed an up-regulation of the expression of *C3* and *Emr1*. Both of these genes are expressed in microglia and, among murine peripheral immune cells, specifically in monocytes/macrophages, but injury induces *C3* expression also in other cell types of the brain [reviewed by Stephan *et al.* (20)]. Activation of complement proteins has been demonstrated to occur spontaneously following human ischemic and hemorrhagic stroke (21). Whereas these gene expression changes were not accompanied by a clear increase in phagocyte number in the peri-infarct cortex, AAV7-MANF did have a robust increasing effect on the number of macrophages in both the external capsule and dorsal striatum. It is unclear whether these cells were microglia- or peripheral monocyte-derived. The effect of MANF on macrophages seems to be transient because the number of phagocytic cells around the infarct was not different at a later time point of day 16 after injury. The effect of overexpressed MANF on innate immune cell recruitment is in agreement with what has been reported after retinal damage (9), but unlike what the authors of that report proposed, our data suggest that, during recovery from cerebral ischemic injury, MANF

is not selectively recruiting alternatively activated macrophages. Notably, it remains unclear whether this effect of overexpressed MANF is shared by endogenous MANF because genetic reduction of endogenous MANF levels in the CNS did not influence the expression of *C3* and *Emr1*. Alternatively, despite the great reduction in the overall levels of MANF in the brain tissue, the postulated autocrine effect of MANF in immune cells (9) might be unaffected in our *Nestin^{Cre/+}::MANF^{fl/fl}* animals, where *Manf* is deleted only in cells of neural origin.

It is unclear whether the effect of AAV7-MANF on immune cells is direct. For example, *C3* not only can attract immune cells but can also influence their phagocytic activity (20). The increased phagocyte number could lead to faster tissue repair through faster clearance of cellular remnants such as the neurite outgrowth-inhibiting myelin debris (22). This could perhaps aid in the repair and rewiring of damaged fiber tracts in the external capsule and dorsal striatum. Nevertheless, it is unclear whether the fast onset of behavioral recovery can be explained by the effect of MANF on immune cell-mediated tissue repair alone. For example, depletion of monocyte-derived macrophages during the first days after injury did not manifest in tests of behavioral recovery until several weeks after injury (23), but the ischemic injury and behavioral tests used in the study differ from ours, making a direct comparison difficult.

Finally, we recognize the limitations of our study as rodent stroke studies have been criticized for poor translatability of their findings to the clinic. We have used young healthy male animals that do not represent the heterogeneous, aged patient population that is most commonly affected by stroke and suffers from multimorbidity. In the future, it would be important to confirm the therapeutic effect of MANF in female and old animals, as well as in thromboembolic models. In addition, future studies should set out to clarify the mechanism behind the recovery-promoting effect of MANF.

In conclusion, we found that delayed poststroke delivery of hMANF was able to promote functional recovery of rats. From a clinical point of view, it is of critical importance that the pharmacological treatment can be administered after stroke. Therefore, MANF or small-molecule MANF mimetics could serve as a potential pharmacological means to boost the functional recovery of stroke patients.

MATERIALS AND METHODS

Study design

In all experiments, all rats and samples were labeled with a number code that did not indicate the treatment, and behavioral tests were carried out by a blinded investigator. The first neurorestoration study with AAV7-MANF was designed to study whether delayed intracerebral AAV7-MANF delivery after dMCAo enhanced the reversal of ischemic injury-induced behavioral deficits in rats (a controlled laboratory experiment with a prespecified hypothesis), as measured by BNST, EBST, cylinder test, and a test for spontaneous horizontal and vertical motor activity. The rats were allocated to two treatment groups (AAV7-MANF and AAV7-GFP) so that the average severity of injury-induced behavioral deficits, as assessed by BNST and EBST on the day before treatment, was equal between the two groups. None of the animals that survived the surgeries were excluded, and there were no outliers removed.

The second neurorestoration study with AAV7-MANF was designed to study whether delayed intracerebral AAV7-MANF delivery to rats after dMCAo influenced the development of the ischemic lesion, as assessed by MRI (a prespecified hypothesis), and to provide samples for immunohistochemical analysis of brain tissue. The specific antigens

analyzed were selected after the initiation of data collection. This study also tested whether the initial finding of MANF's recovery-enhancing effect could be reproduced in BNST and EBST and whether the treatment effect could also be seen in comparison to a second control group of rats treated with buffered saline solution. The rats were allocated into three treatment groups (AAV7-MANF, AAV7-GFP, and PBS) so that the average infarct volume, as assessed by T2-weighted MRI immediately before treatment, and behavioral test scores were equal between the three groups. Two rats were excluded before the treatment because they had lesions too large or too small. Three rats were excluded (one from each group) before the analysis of results because they had large lesions that were not confined to the cortical area. Two rats were excluded because in subsequent analysis, GFP transgene expression could not be detected in their brains. No other animals that survived the surgeries were excluded, and there were no other outliers removed.

The neurorestoration study with chronic rhMANF was designed to study whether another mode of hMANF delivery after dMCAo also enhanced the reversal of ischemic injury-induced behavioral deficits in rats (a controlled laboratory experiment with a prespecified hypothesis), as measured by BNST, EBST, and the cylinder test. The rats were allocated into three treatment groups based on the severity of their neurological deficit assessed by BNST on day 2 after dMCAo surgery.

The third neurorestoration study with AAV7-MANF was designed to characterize the speed of behavioral effect onset in BNST and EBST after intracerebral AAV7-MANF delivery to rats after dMCAo (a prespecified hypothesis) and to provide samples for transcriptome analysis of peri-infarct tissue. The rats were allocated to two treatment groups (AAV7-MANF and AAV7-GFP) so that the average severity of injury-induced behavioral deficits, as assessed by BNST and EBST on the day before treatment, was equal between the two groups. A separate set of animals, treated with AAV7-MANF or AAV7-GFP ($n = 7$), was used to provide samples for immunohistochemical analysis of brain tissue. One rat was excluded from the analysis because it had a very small lesion.

Production of viral vectors

Self-complementary AAV vectors expressing hMANF (dsAAV7-MANF) or GFP (dsAAV7-GFP) were generated and purified as described previously (6).

Distal middle cerebral artery occlusion

All animal experiments were approved by the Finnish National Ethics Board and carried out according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Ligation of the right MCA and bilateral common carotids (CCAs) was performed on male Sprague-Dawley rats (Harlan) using methods previously described (7). Briefly, the bilateral CCAs were identified and isolated through a ventral midline cervical incision. Rats were placed in a stereotaxic apparatus, and a craniotomy was carried out over the right hemisphere. The right MCA was ligated with a 10-0 suture, and bilateral CCAs were ligated with nontraumatic arterial clamps. After 60 or 90 min of ischemia, the suture around the MCA and arterial clips on CCAs were removed. After recovery from anesthesia, the rats were returned to their home cage.

Behavioral tests

BNST, EBST, and horizontal and vertical motor activity (Med Associates Inc.) test were carried out as described previously (7). In the latter, the distance moved and the number of vertical activity counts during 60 min were expressed in relation to the activity of the same individual on day 1 after the surgery. Cylinder test was carried out as described pre-

viously (24). In the cylinder test, the animal was placed inside a transparent vertical tube with a diameter of 35 cm, and its movement was video-recorded for 5 min. The number of first front paw touches to the inner wall of the tube, after rising on hindlimbs, was counted. In all of the tests, the investigator was unaware of the treatment each animal had received.

First and third neurorestoration studies with AAV7-MANF

Male Sprague-Dawley rats (280 to 320 g in the first study and 200 to 280 g in the third study) were anesthetized with intraperitoneal chloral hydrate injection (0.4 g/kg) and underwent a 60-min dMCAo surgery. One day later, the animals were examined using the BNST and EBST. On the basis of the scores, the animals were then divided into two groups, keeping the average score of the two groups equal. On day 2 after dMCAo surgery, the animals were anesthetized with isoflurane and attached to a stereotaxic frame. AAVs were injected intracerebrally into two subcortical sites with the following stereotaxic coordinates: site 1, anteroposterior (A/P) +1.6, lateral-medial (L/M) +2.2, and dorso-ventral (D/V) -5.0 (from the surface of the skull); site 2, A/P -0.4, L/M +4.0, and D/V -5.0. Two and a half microliters of AAV7-GFP (titer, 1.1×10^{13} vg/ml) or AAV7-hMANF (8.1×10^{12} vg/ml) was injected at a speed of 1 μ l/min using a 10- μ l Hamilton syringe with a 30-gauge blunt needle. The needle was kept in place for 2 min after the injection. In the first study, the animals were tested in the cylinder test, BNST, and EBST on days 7 and 14 after the dMCAo surgery. In addition, horizontal and vertical motor activity was assessed on day 14, after which the animals were sacrificed for the collection of tissue material. In the third study, the rats were tested with BNST and EBST on day 4 and sacrificed for the collection of tissue material.

Second neurorestoration study with AAV7-MANF

Male Sprague-Dawley rats (250 to 290 g) were anesthetized with intraperitoneal chloral hydrate injection (0.4 g/kg) and underwent a 60-min dMCAo surgery. Two days later, the animals were anesthetized with isoflurane, underwent MRI, and were divided into three groups based on the lesion volume measured from T2-weighted images. After MRI, the animals were injected intracerebrally with AAVs, as described above. The rats were also imaged on day 9 and 16 days after dMCAo and examined with BNST and EBST on day 16 after the dMCAo surgery. After that, the rats were perfused transcardially with 0.9% NaCl solution, followed by 4% paraformaldehyde (PFA) in PBS. The brains were dissected out and postfixed for 2 to 4 days in 4% PFA in PBS. After dehydration and clearing with xylene, the brains were embedded in paraffin wax and sectioned.

Neurorestoration study with chronic rhMANF delivery

Male Sprague-Dawley rats (220 to 260 g) underwent 90-min dMCAo, and infusion cannulas, connected to subcutaneously placed osmotic mini pumps (ALZET Model 2002, Durect Corp.), were implanted into the peri-infarct region (coordinates: A/P, -0.5; L/M, +1.9; D/V, -2.5) and secured to the skull using three stainless steel screws and polycarboxylate cement (Aqualox, VOCO). rhMANF (3 μ g/day) or PBS (pH 7.4) (vehicle-treated group) was infused (0.25 μ g/ μ l, 0.5 μ l/hour) from days 3 to 16, after which the pumps were removed. The animals were humanely sacrificed 10 days later. Behavioral tests were conducted on days 2, 7, 14, and 24 (the cylinder test was not performed on day 7).

Statistical analysis

The significance of treatment-induced differences in BNST and EBST was assessed with the Mann-Whitney *U* test (two-tailed) when two

groups of rats were compared. For the comparison of three treatment groups, the Kruskal-Wallis test was used, followed by Dunn's multiple comparisons post hoc test. One-way ANOVA and Tukey's multiple comparisons post hoc test were used for the comparison of differences in average infarct size, qPCR results, and parameters quantified from immunohistochemical stainings. Two-tailed *t* test was used when comparing only two groups. Statistical significance was considered at $P < 0.05$.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/4/5/eaap8957/DC1>

Supplementary Materials and Methods

fig. S1. The expression patterns of MANF and GFP 2 weeks after intracerebral AAV7 injection.

fig. S2. Postischemic AAV7-MANF injection does not affect spontaneous motor activity.

fig. S3. AAV7-MANF injection after ischemia does not reduce lesion size.

fig. S4. Analysis of anti-laminin and anti-synaptophysin immunoreactivity in the peri-infarct area.

fig. S5. Analysis of anti-MBP immunoreactivity in the peri-infarct area.

fig. S6. Analysis of anti-gliial fibrillary acidic protein immunoreactivity in the peri-infarct area.

fig. S7. AAV7-MANF injection after ischemia results in rapid hMANF expression and enhancement of behavioral recovery.

fig. S8. Analysis of MBP and CD68 colocalization in the external capsule.

fig. S9. Analysis of immune cell number on day 14 after AAV7-MANF treatment.

fig. S10. qPCR analysis of alternative activation marker *TGFβ1* and *Mrc1* (*CD206*) mRNA levels in the peri-infarct cortex at day 4 after ischemic injury ($n = 4$ to 5).

fig. S11. Endogenous MANF has no effect on *C3*, *Emr1*, *Arg1*, or *Mrc1* expression in the cortex after cerebral ischemic injury.

table S1. Top hits from RNA sequencing analysis performed to find genes expressed differentially in the peri-infarct region of AAV7-GFP-treated ($n = 3$) versus AAV7-hMANF-treated ($n = 3$) rats.

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