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ORIGINAL ARTICLE

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Overexpression of Adiponectin Improves Neurobehavioral Outcomes After Focal Cerebral Ischemia in Aged Mice

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Keywords
Adiponectin; aged mice; angiogenesis; cerebral ischemia; neurobehavioral recovery.

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SUMMARY

Aims: To study whether adiponectin (APN) could improve neurological outcomes in aged mice after ischemic stroke. **Methods:** Adeno-associated virus carrying APN gene was injected into aged and young adult mice 7 days before transient middle cerebral artery occlusion (tMCAO). Atrophic volumes and neurobehavioral deficiencies were determined up to 28 days after tMCAO. Focal angiogenesis was determined based on blood vessel number in the ischemic regions. **Results:** Increased atrophic volume and more sever neurobehavioral deficits were found in the aged mice compared with young adult mice ($P < 0.05$). AAV-APN gene transfer attenuated atrophic volume and improved neurobehavioral outcomes, along with increased focal angiogenesis in both aged and young adult mice, compared with control animals ($P < 0.05$). In addition, the attenuation of atrophic volume and the improvement in neurobehavioral outcomes were much more significant in aged mice than in young adult mice after AAV-APN administration ($P < 0.05$). The number of microvessels in aged AAV-APN mouse ischemic brain was higher than in young adult AAV-APN treated mouse brain ($P < 0.05$). **Conclusions:** Our results demonstrate that APN overexpression reduces ischemic brain injury and improves neurobehavioral function recovery in aged mice than in young mice, suggesting APN is more beneficial in aged animals after ischemic stroke.

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The first two authors contributed equally to this work.

Introduction

Ischemic stroke is one of the most vital disorders with high mortality and morbidity in China and worldwide [1]. Over the last two decades, numerous neuroprotective drugs were proven to be effective for treating acute stroke in animal stroke models [2,3]. However, none of these drugs were effective in subsequent clinical trials [4]. Studies showed that the efficacy of a drug varied in different experimental stroke models. The relationship between the efficacy of drugs and its mechanism remains inconsistent [5]. In addition, aging is one of the most important factors in influencing the result of drug because the efficacy of drug is totally different in young adult and aged human or experimental animals [6]. Moreover, the aging process is related to cellular functions, and aging attenuated ischemia-induced angiogenesis [7,8]. Consistent with these observations, in models of heart disease and both global and focal cerebral ischemia, the prominence of ischemic changes advances with age [9–11], as are postischemic behavioral abnormalities [12]. Therefore, using aged animal models of ischemic

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stroke to assess drugs is essential for the cerebral ischemia research and for clinical translation.

Adiponectin (APN), an adipose-specific plasma protein, plays a protective role in the development of cardiovascular morbidity [13,14]. APN ameliorated endothelial function and modulated inflammation [15]. High level of APN in peripheral blood is associated with a reduced risk of cardiovascular diseases such as the coronary artery disease and the myocardial infarction, while low plasma APN was related to an increased risk of 5-year mortality after first-ever ischemic stroke [16,17]. APN suppressed the development of atherosclerosis by inhibiting smooth muscle cell proliferation and migration, which could be related to the vascular protective activity [18,19]. APN also promotes angiogenesis by up-regulating AMPK and Akt signaling in endothelial cells or through endothelial nitric-oxide-synthase-dependent mechanism [20–22]. Our previous study demonstrated that APN overexpression attenuated brain atrophic volume, improved neurobehavioral recovery, and promoted cerebral angiogenesis 14 days after tMCAO in young adult mice. The effect of APN was mediated by activating AMPK signaling pathway [23]. However, whether the similar effects of APN occur in aged brain remains to be further investigated.

Recent studies documented that angiogenesis could be induced after focal cerebral ischemia in animal and human brains [24–26]. Stroke patients with a higher density of microvessels are associated with less morbidity and longer survival [27–29]. Cerebral ischemia-induced angiogenesis showed benefits for the recovery of motor function [30–32]. These observational studies indicate that focal angiogenesis and neovascularization play important roles for ischemic brain repairing and remodeling. However, whether APN promotes angiogenesis in aged brain remains unknown.

In this study, we aim to explore whether APN is able to overexpress in aged brain via AAV-APN gene transfer, and whether APN overexpression in aged mice has the similar salutary effects as does in young adult mice following cerebral ischemia. In addition, we also ask whether the role of APN in aged mice is different from young adult mice in cerebral ischemia.

Methods

Experimental Groups

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Shanghai Jiao Tong University, Shanghai, China. To compare endogenous APN expression in aged and young adult mice brain after stroke, tMCAO was performed in aged male CD-1 mice (22–24 month-old, Ship BK, Shanghai, China, n = 6, 3 for Western blot and three for immunostaining; n = 6 in sham group, three mice for Western blot and three mice for immunostaining) and young adult male CD-1 mice (3-month-old, grouped the same way as aged mice). To evaluate the efficiency of AAV-APN gene transfer in normal mice brain, aged mice group and young mice group were received AAV-APN transfer (n = 6 per group, three mice for Western blot and three mice for immunostaining) and AAV-GFP was injected as the control group (grouped the same way as AAV-APN injected mice). To test the therapeutic efficiency of APN in ischemic mice brain, AAV-APN was injected into aged and young adult mice brain (n = 10 per group) and AAV-GFP was injected as a control group (n = 10 per group).

AAV-APN Gene Transfer in the Mouse Brain

Aged male CD-1 mice and young adult male CD-1 mice were anesthetized intraperitoneally using ketamine/xylazine (100/10 mg/kg, Sigma) and then placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). Five-microliter viral suspensions containing 4×10^9 genome copies of AAV-APN were injected into the striatum (AP: −1.0 mm; L −2.0 mm; V −2.5 mm) at a rate of 0.2 μL/min based on our previous study [33]. The needle was withdrawn 25 min after injection, and animals were allowed to return to the home cage after mice wakened. A group of mice underwent AAV-GFP gene transfer as a viral vector control.

Transient Middle Cerebral Artery Occlusion (tMCAO) in Mice

tMCAO was performed 7 days after AAV-APN gene administration as previously described [23,34]. Briefly, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated by a surgical microscope (Leica, Wetzlar, Germany). A silicone-coated 6-0 suture was gently inserted from ECA stump to ICA to occlude the opening of MCA. The success of occlusion was determined by monitoring the decrease in surface cerebral blood flow (CBF) to 10% of baseline CBF using a laser Doppler flowmetry (Moor Instruments, Devon, England). Reperfusion was performed by the suture withdrawal after 90 min of tMCAO. PH, partial pressure of carbon dioxide (pCO_2), and partial pressure of oxygen (pO_2) were measured using i-STAT® System (Abbott Point of Care Inc. Princeton, NJ, USA), and blood pressure was determined by Softron® Sphygmomanometer (Softron BP-98A, Softron Beijing Inc. Beijing, China). The mice in which CBF dropped to less than 90% of baseline immediately after MCAO and those died during surgery were excluded in the stroke cohorts.

Brain Atrophy Measurement

Brains were removed and frozen immediately in −40°C isopentane. Twenty-micrometer-thick section was cut from the frontal pole to hippocampus and stained with 0.1% cresyl violet (Sinopharm Chemical Reagent Co., Shanghai, China). Brain atrophic volume was analyzed using NIH Image J software as previously described [23] and calculated by the following formula: contralateral hemisphere minus normal region of ipsilateral hemisphere, then multiplied by the section interval thickness.

Neurobehavioral Tests

Mice were trained for three consecutive days prior to surgery. Neurobehavioral tests were performed before and 1, 3, 7, 14, and 28 days after tMCAO by an investigator who was blinded to the experimental groups. Modified neurological severity scores

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(mNSS) of the animals were graded on a scale of 0–14, which is a composite of motor, reflex, and balance tests [35].

For rotarod test, mice were placed on an accelerating rotarod cylinder (Zhenghua, Anhui, China); the speed was increased from 20 to 40 rpm within 5 min. The trial ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rungs. The time that animals remained on the rotarod was recorded for further analysis [24].

For beam-walking test, mice were trained to traverse a horizontally elevated square beam with 7 mm in diameter to reach an escape platform placed one meter away. Mice were placed on one end of the beam, and the latency to traverse 80% of the beam toward the escape platform was recorded from three independent trials.

Asymmetric motor behavior was also performed using the corner test. Mice were placed between two boards with dimensions $30 \times 20 \times 1$ cm^3 for each in home cage [36]. Normal animals turn back randomly from either left or right. However, ischemic animals preferentially turn toward the impaired side. The number of turns taken on each side was recorded from 10 trials of each test.

Western Blot Analysis

Tissue sample was collected from the ipsilateral hemisphere, including injured cortex and striatum, and quantified with BCA protein assay (Pierce, Rockford, IL, USA). Protein (30 μg) was separated by 10% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Whatman, Piscataway, NJ, USA). After blocking with 5% skim milk, the membrane was probed with anti-APN antibody (1:500 dilution; R&D, Minneapolis, USA) and visualized using an ECL system (Thermo, Rockford, CA, USA). Image was taken and calculated by Quantity One software (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Frozen brain sections were fixed in 4% paraformaldehyde for 10 min and then blocked with 10% BSA. Sections were incubated overnight at 4°C with CD31 (1:200 dilution, R&D), NeuN (1:100 dilution, Millipore. Rockland, Massachusetts, USA), GFAP (1:300 dilution, Millipore), alpha smooth muscle actin (1:300 dilution, R&D), and PCNA (1:200 dilution, Abcam, Cambridge, MA, USA). After washing, sections were further stained by 488-conjugated and Cy3-conjugated antibody (1:1000 dilution, Jackson Immuno Research, West Grove, PA, USA), as previously described [37]. Sections were examined under Leica TCS-SP5 microscope (Leica, Solms, Germany). Images were acquired with LAS AF Software (Leica) using 488 nm or 594 nm excitation laser wavelength, and the exposure time was about 735 ms.

Microvessel Counts

The brain regions that located at left, right, and bottom areas of the needle track from each mouse were chosen. Two investigators blinded to the experimental group assessed blood vessel number separately. Only microvessels with a clearly defined lumen or a well-defined linear vessel shape were taken into account. Single endothelial cells were ignored. The number of blood vessels was calculated as the mean of the blood vessel counts obtained from the six pictures as previously described [38]. The number of small arteries was calculated in the same way.

Statistical Analysis

Data were presented as mean ± SD. Comparison of two groups was analyzed by an unpaired Student's *t*-test. Three group comparison data were analyzed by one-way ANOVA with Dunnett's test. Mortality rates were compared by the chi-square test. A probability value of less than 5% was accepted as statistical significance.

Results

Increased APN Expression in Aged Mouse Brain After tMCAO

Western blotting and immunohistochemistry were performed to determine the expression profiles of APN in aged and young adult mouse brains after tMCAO. We found that APN was low in normal mouse brain, while the expression was increased in the ischemic mouse brain, which was mainly located near small vessels of ischemic brain. APN expression was also increased in aged mouse brain as early as 1 day after tMCAO and persisted up to 7 days. It was noted that APN expression in the ischemic brain of young adult mouse was significantly higher than that in the aged mouse brain at 1, 3, and 7 days after ischemia (Figure 1A–C).

APN Overexpression in Aged Mouse Brain After AAV-APN Injection

To determine the success of gene transfer, we examined the extent of GFP expression after AAV-GFP gene transfer. We revealed that the GFP expression could be detected in aged mouse brain for at least 3 weeks (Figure 2A,B). Western blot analysis showed that APN expression was significantly increased in the ipsilateral hemisphere in AAV-APN-treated aged mice after tMCAO ($P < 0.05$). The APN level reached plateau at day 7 and sustained for at least 21 days (Figure 2C,D). Expression pattern of APN in aged mouse brain is similar to that in young adult mouse brain. Double immunostaining demonstrated that APN was expressed in endothelial cells, neurons, and astrocytes after AAV-APN transfer (Figure 2E).

APN Overexpression Attenuated Atrophy in Aged Mice

To explore the effect of APN on the histological outcome after ischemic injury, whole-brain atrophic volume was examined 2 weeks after tMCAO (Figure 3A). We demonstrated that the atrophic volume was significantly increased in the aged mouse brain compared with that in the young adult mouse brain. In addition, atrophic volume 2 weeks after tMCAO was greatly attenuated in aged mice after AAV-APN gene transfer compared with the control group (Figure 3B, $P < 0.05$). Interestingly, the extent of attenuated brain atrophy in aged mice was greater than

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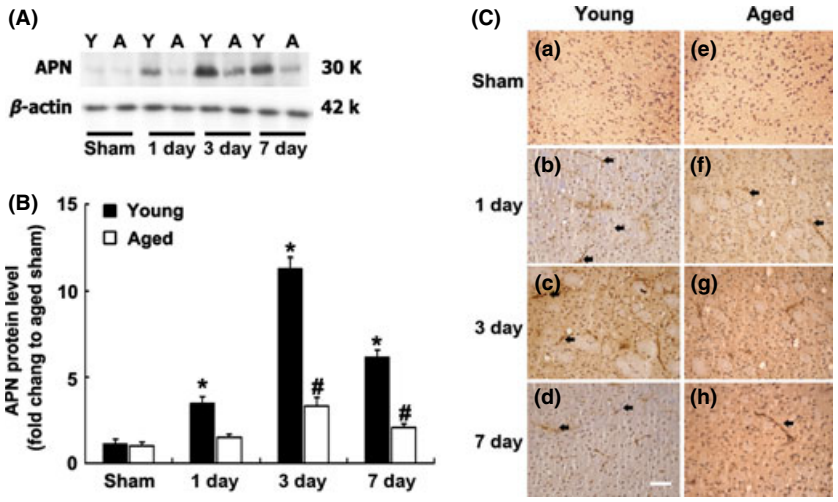

Figure 1 APN was increased in ischemic brain of aged mice. (**A**) Western blot analysis showed APN expression in normal and ischemic aged and young adult mouse brain at different durations after tMCAO. Y: young mice; A: aged mice. (**B**) Bar graph showed semi-quantitative APN expression from (**A**). Data are presented as mean ± SD, N = 3 per group. $^{*}P < 0.05$, APN-young adult versus APN-aged group; $^{\#}P < 0.05$, APN-aged versus APN-aged sham group. (**C**) Photomicrographs showed the expression of APN in both sham (a, e) and ischemic aged and young adult mouse brain at 1 (b, f), 3 (c, g) and 7 days (d, h) after tMCAO. N = 3 per group. Arrows indicate the APN signal. Bar = 50 μm.

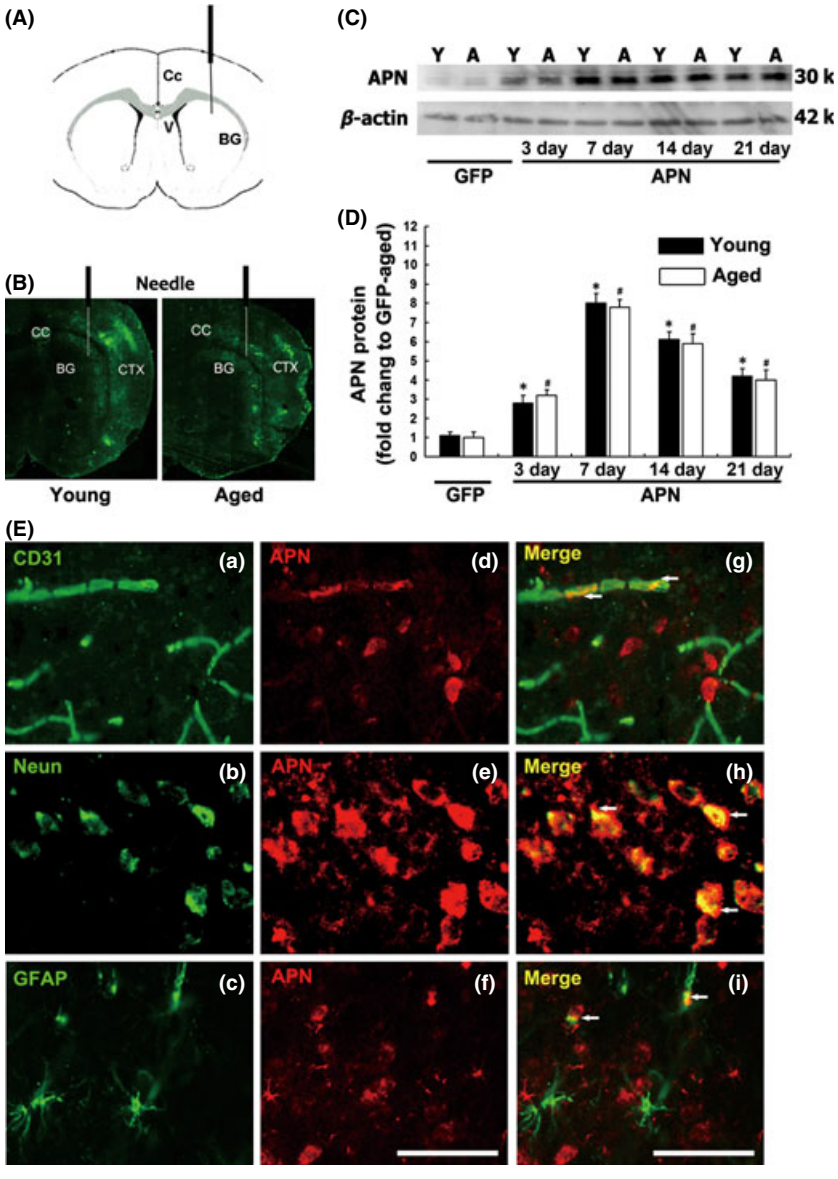

Figure 2 APN overexpression in aged mouse brain after AAV-APN gene transfer. (**A**) Graphic illustration indicated injection point in a mouse brain coronal section. (**B**) The distribution of GFP expression in aged (a) and young adult (b) mouse brain three weeks after AAV-GFP gene transfer. (**C**) Western blot analysis showed APN expression in aged and young adult mouse brain after 3, 7, 14, and 21 days of AAV-APN transduction. (**D**) Bar graph showed semi-quantitative APN expression. Data are presented as mean ± SD, N = 3 per group. $^{*}P < 0.05$, APN-young adult versus GFP-young adult group; $^{\#}P < 0.05$, APN-aged versus GFP-aged group. (**E**) Photomicrographs showed that APN was expressed in endothelial cells (a, d, g), neurons (b, e, h), and astrocytes (c, f, i) after AAV-APN transduction. Bar = 50 μm.

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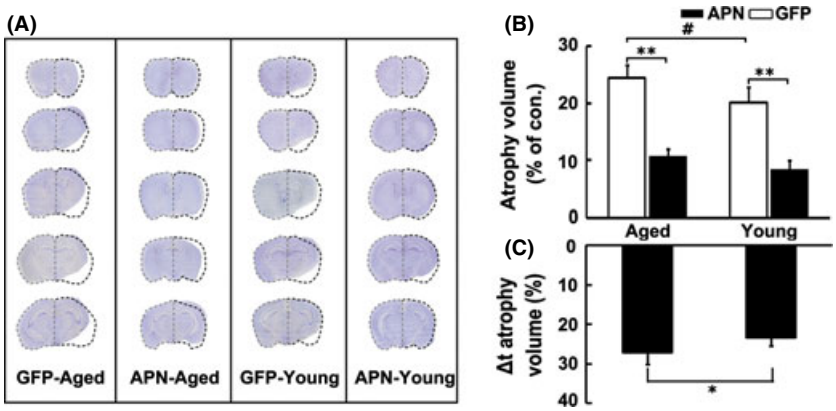

Figure 3 APN overexpression attenuated brain atrophy in aged mice after tMCAO. (**A**) Photographs represented cresyl violet staining of coronal sections from AAV-APN-transduced aged and young adult mice following 14 days of tMCAO. Dash lines illustrated the atrophic areas compared with the contralateral hemisphere. Bar graph showed that total atrophic volume in the AAV-APN transduced aged and young adult mice (**B, C**). Data are mean ± SD. N = 10 in each group. $^{\#}P < 0.05$, GFP-aged versus GFP-young adult groups; $^{**}P < 0.01$, APN versus GFP groups; $^{*}P < 0.05$, APN-aged versus APN-young adult groups.

in young adult mice (30 vs. 20%, Figure 3C, Δt atrophic volume, $P < 0.05$), suggesting that APN exerts its protective effect more efficiently in aged ischemic mice.

APN Improved Neurobehavioral Recovery After tMCAO in Aged Mice, While Did Not Affect Mice Mortality

To determine whether APN overexpression could improve neurobehavioral outcomes as it does in young adult mice, neurobehavioral tests were performed in aged mice with AAV-APN gene or vehicle injection. We proved that motor function based on the neurological score, beam walk test, rotarod test, and corner test was greatly improved at 7, 14, and 28 days after AAV-APN administration following tMCAO, compared with the control group (Figure 4, $P < 0.05$). More severe neurobehavioral impairments were detected in aged mice after tMCAO. Remarkably, the magnitude of neurobehavioral recovery was greater in aged mice than in young adult ischemic mice (Figure 4E–H, Δt neurobehavioral tests, $P < 0.05$).

Cerebral blood flow, mean arterial blood pressure (MABP), PH, pCO_2, and pO_2 were recorded, and they were similar between the groups (Table S1). In addition, APN injection did not affect the mortality rate after stroke (Table S2).

APN Stimulating Focal Angiogenesis in Aged Mice After tMCAO

To determine whether APN promoted focal angiogenesis after tMCAO, we counted the number of microvessels in ischemic perifocal region (Figure 5A). The number of microvessels was increased in aged ischemic mice injected with AAV-APN gene compared with the control (Figure 5B, $P < 0.05$) and the young adult mice (Figure 5C, $P < 0.05$). PCNA and CD31 double staining demonstrated that the number of proliferating endothelial cells was increased in ischemic perifocal region in aged mice with AAV-APN gene transfer (Figure 5D, $P < 0.05$), suggesting that aged brain retained the capacity of angiogenesis in response to ischemic injury. Similarly, angiogenesis was increased in aged mice more than in the young adult mice 4 weeks after APN-AAV treatment following tMCAO (Figure 5E, Δ number of newly formed microvessels, $P < 0.05$).

To determine whether small arteries in the ischemic brain were also increased after APN overexpression, we further examined the number of smooth muscle cells in perifocal region. We found that the number of αSMA-positive cells was greatly increased in aged ischemic brain after AAV-APN gene transfer, compared with the control (Figure 5F,G, $P < 0.05$). Similarly, the increased number of small arteries in aged mice was greater in young adult mice 2 weeks after tMCAO (Figure 5H, Δnumber of small arteries, $P < 0.05$), suggesting APN not only promotes angiogenesis, but also improves focal neovascularization.

Discussion

In this study, we demonstrated that APN could be overexpressed in the aged mouse brain under both normal and ischemic conditions. APN overexpression not only reduced the ischemic brain injury and promoted neurobehavioral outcomes in aged mice, but also displayed even better therapeutic effects compared with those in the young adult mice. In addition, focal angiogenesis was significantly increased in the aged ischemic brains after AAV-APN gene transfer. Our findings suggest that APN is a potential therapy target for ischemic brain injury, especially in the aged mice.

We reported that APN overexpression via AAV-APN gene transfer could greatly reduce ischemic brain injury and promote neurobehavioral recovery in young adult mice [23]. However, whether APN has similar functions in the aged recipients remains unknown. It is possible that aged brain is less responsive to APN treatment because aging adversely influences stroke outcomes due to age-related changes in the brain microenvironment [39,40]. For example, several growth factors, such as VEGF and IGF-1, which stimulate angiogenesis and neurogenesis, reduce with aging [41,42]. Besides, aging reduces capillary density after

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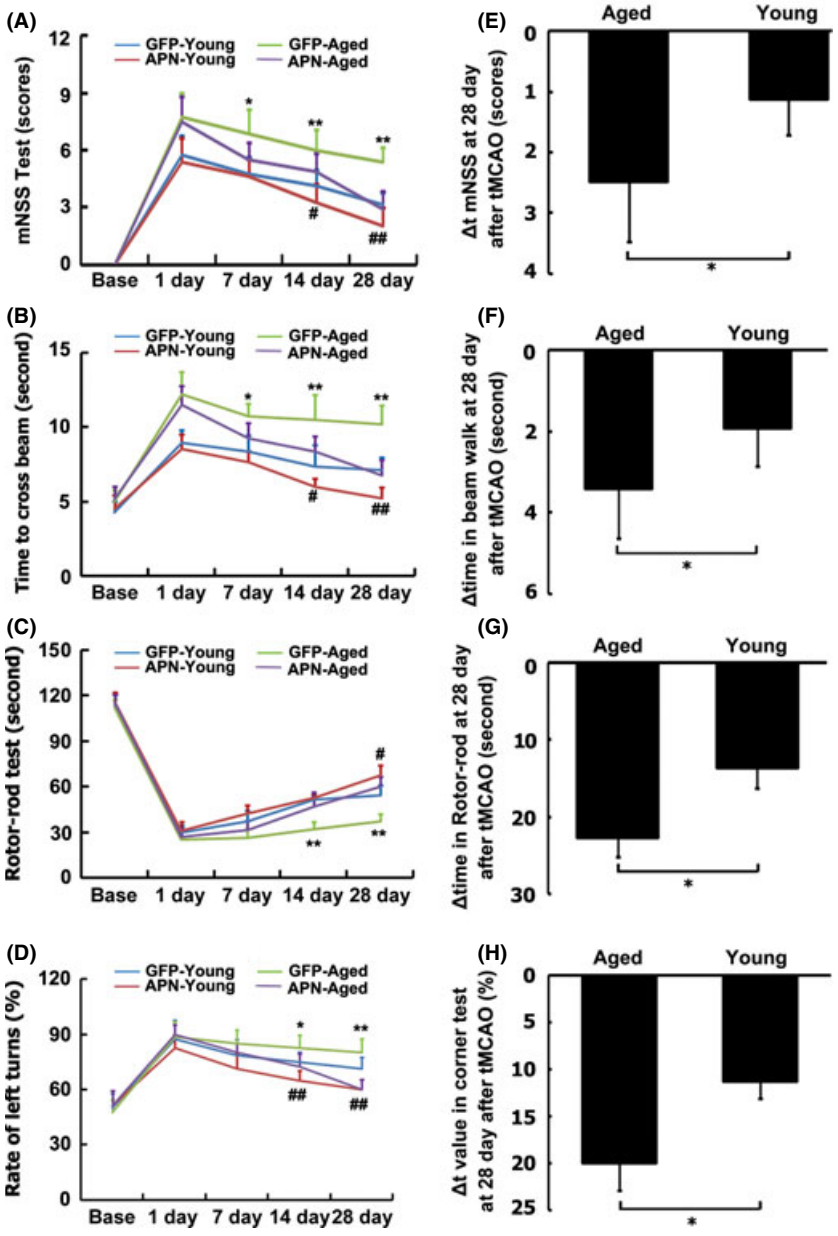

Figure 4 APN overexpression improved neurobehavioral recovery in aged mice after tMCAO. Neurobehavioral tests were evaluated using neurological score (**A**), beam walk test (**B**), rotarod test (**C**), and corner test (**D**). The behavior tests were performed at 1 day before tMCAO, 1, 3, 7, 14, and 28 days after tMCAO. Data are mean ± SD, n = 10 per group. *or **$P < 0.05$ or $P < 0.01$, aged APN versus aged GFP groups, # or ##$P < 0.05$ or $P < 0.01$, young adult APN versus young adult GFP groups. Δ of neurological score (**E**), beam walk test (**F**), rotarod test (**G**), and corner test (**H**) was analyzed between aged APN and young adult APN group. *$P < 0.05$, aged APN versus young adult APN group.

hindlimb ischemia in New Zealand white rabbits [43]. Angiogenesis is also reduced wound-healing process in aged rats [44]. If the down-stream signals of APN are these neurotrophic factors, the APN treatment in the aged brain would be futile. Nevertheless, our present results demonstrated that the effects of APN in aged mice were even better than those in young adult mice, suggesting APN treatment could be used for the treatment of aged-related diseases.

Our previous studies demonstrated that when injecting AAV vector into the lateral caudate putamen, overexpression of target genes could be achieved in both the parenchyma and ependymal tissues in the young adult mice [23]. Whether AAV vector induced target gene overexpression in aged mice was unknown. In the present study, we found that APN overexpression was similar in aged mice and young adults, which reached the maximum in both young adult and aged mice 7 days after AAV-vector injection and sustained for at least 3 weeks, demonstrating that AAV vector was capable of maintaining a high level of APN in aged mouse brain. Aging is not a barrier for the gene therapy.

Gene therapy has some limitations for its clinical translation. For instance, injecting targeted gene directly into the brain is invasive and repeated injection is not allowed. These problems hamper its translation from bench to bedside. For better translation into clinic, several strategies could be developed. For example, intravenous injection of TAT fusion protein sufficiently permeates the blood brain barrier [45,46]; thus, APN protein fused to TAT could

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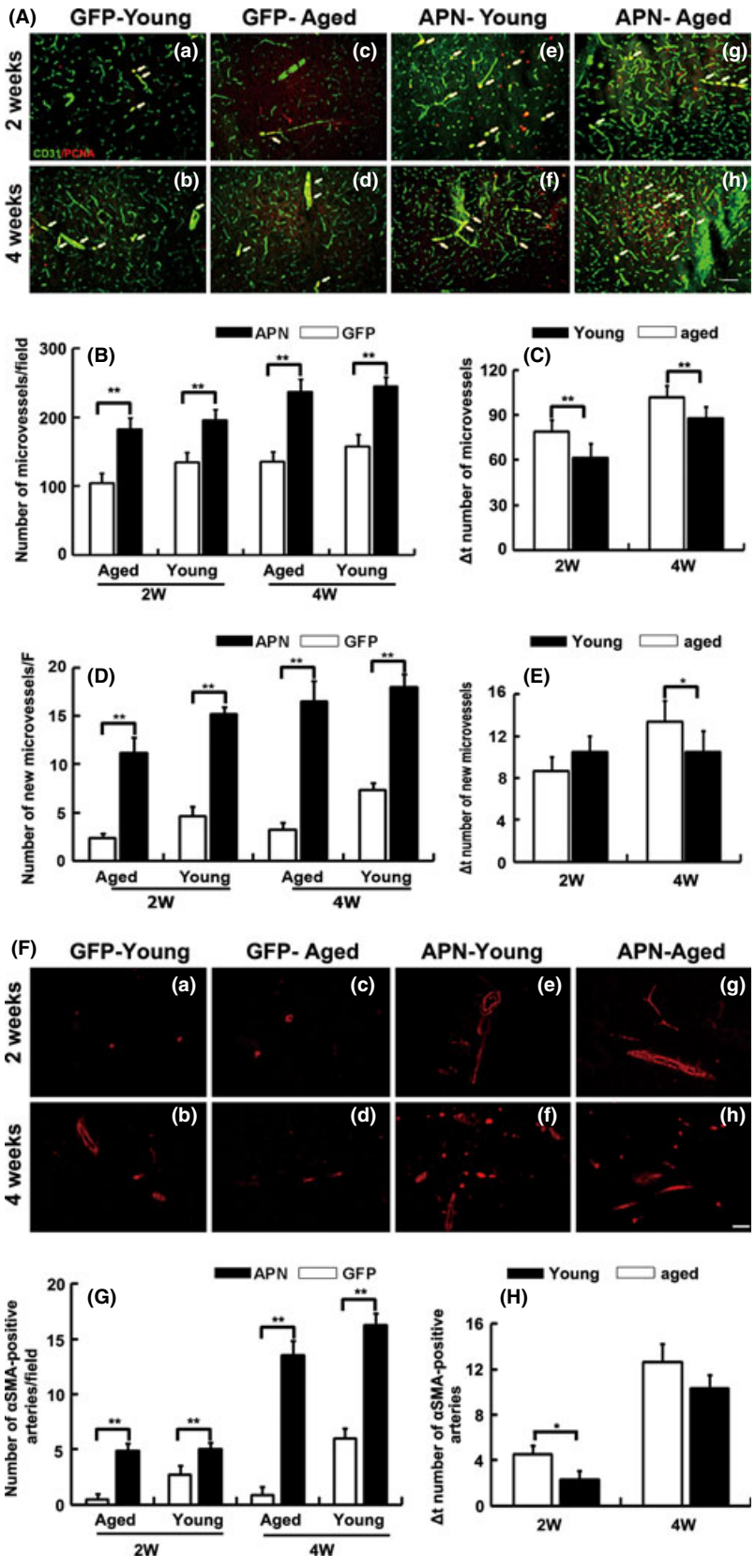

Figure 5 Angiogenesis was increased in aged mice with AAV-APN gene transfer after tMCAO. (**A**) Photomicrographs showed the CD-31 and PCNA double immunostaining in perifocal region in AAV-APN transduced aged mouse brain 2 and 4 weeks after tMCAO. AAV-APN transduced young adult mice and AAV-GFP transduced aged mice were as control. Bar = 20 μm. (**B**) Bar graph showed the number of microvessels in AAV transduced aged and young adult mice. Values are mean ± SD, N = 6 in each group. $^{**}P < 0.01$, APN versus GFP groups. (**C**) Bar graph showed that the number of microvessels between aged APN and young adult APN groups. $^{**}P < 0.01$, aged APN versus young adult APN group. (**D**) Bar graphs showing the number of newly formed microvessels in the AAV-APN transduced aged mice. Data are mean ± SD, n = 6 per group. $^{**}P < 0.01$, APN versus GFP groups. (**E**) Bar graph showed that the number of microvessels between aged APN and young adult APN groups. Data are mean ± SD, n = 6 per group. $^{*}P < 0.05$, aged APN versus young adult APN group. (**F**) Photomicrographs showed SMA-positive cells in AAV-APN transduced aged mouse brain 2 weeks and 4 weeks after tMCAO. (**G**) Bar graph showed the number of small arteries in the AAV-APN transduced aged mice after 2 weeks and 4 weeks of tMCAO. Data are mean ± SD, n = 6 per group. $^{**}P < 0.01$, APN versus GFP groups. (**H**) Bar graph showed that the number of small arteries between aged APN and young adult APN groups. Values are mean ± SD, N = 6 in each group. $^{*}P < 0.05$, aged APN versus young adult APN group.

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be injected intravenously into the ischemic patients. In addition, with a combination of stem cell and gene therapy, APN could be delivered into the brain via stem cells. APN could be overexpressed in stem cells, and then stem cells could be injected into the ischemic patients through various administration routes [47–50].

Our major finding is that APN overexpression confers benefits not only in young adult mice, but also in aged ischemic mouse brain. More importantly, the magnitude of reduction in atrophic volume and the extent of neurobehavioral recovery afforded by APN gene transfer in aged ischemic mice were better than those in young adult mice. This effect was correlated with the increase in focal angiogenesis in aged mouse brain after ischemic brain injury. It is unclear why aged mice overexpressing APN have better outcomes compared with the young adult mice. One reason could be aged mouse brain response more sensitively to APN. Nevertheless, the benefits of APN in aged mouse brain needs to be further identified.

Concerted actions of angiogenic molecules are needed during angiogenesis, in which VEGF is the most important factor [51,52]. AAV-APN gene transfer could further promote focal VEGF release in young adult mice. Ischemic stress activates AMPK signaling pathway in the HUVEC culture and in the mouse ischemic hind limb model [53,54]. These results suggest that VEGF stimulated angiogenesis in ischemic tissue through AMPK signaling pathway. Indeed, we confirmed that APN promoted AMPK phosphorylation in young adult ischemic mice. Inhibiting AMPK phosphorylation by compound C significantly attenuated VEGF expression and angiogenesis [23], suggesting that the effect of APN on angiogenesis is related to the AMPK signaling during cerebral ischemia.

In conclusion, we demonstrated that APN overexpression in young adult and aged ischemic mice reduced brain atrophy, improved neurobehavioral recovery, and increased angiogenesis, suggesting that APN is a potential therapy target in aged rodents for ischemic brain injury.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Summary of physiological parameters in mice.
Table S2. Mortality rates of mice after tMCAO within 4 weeks.

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