Research paper

Administration of intramuscular AAV-BDNF and intranasal AAV-TrkB promotes neurological recovery via enhancing corticospinal synaptic connections in stroke rats

Jing Wang a,b,1, Yichen Cai b,1, Jingyi Sun c, Hua Feng d, Xiaoyu Zhu b, Qian Chen b, Feng Gao b, Qingbin Ni e, Leilei Mao b,⁎, Mingfeng Yang b,⁎⁎, Baoliang Sun a,b,⁎

a Medical College of Qingdao University, Qingdao 266021, Shandong, China
b Institute for Neurological Research, The Second Affiliated Hospital; School of Basic Medical Sciences of Shandong First Medical University & Shandong Academy of Medical Sciences, Taian, 271000, Shandong, China
c Department of Spinal Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250021, Shandong, China
d Department of Otolaryngology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, Shandong, China
e Postdoctoral Workstation, Taian Central Hospital, Taian 271000, Shandong, China

ARTICLE INFO

Keywords:
Stroke
BDNF
TrkB
Synaptic plasticity

ABSTRACT

Stroke causes long-term disability in survivors. BDNF/TrkB plays an important role in synaptic plasticity and synaptic transmission in the central nervous system (CNS), promoting neurological recovery. In this study, we performed non-invasive treatment methods focused on intramuscular injection into stroke-injured forelimb muscles, or intranasal administration using adeno-associated virus (AAV) vectors carrying genes encoding BDNF or TrkB. In a permanent rat middle cerebral artery occlusion (MCAO) model, we assessed the effects of combination therapy with AAV-BDNF and AAV-TrkB on motor functional recovery and synaptic plasticity of the corticospinal connections. Our results showed that BDNF or TrkB gene transduced in the spinal anterior horn neurons and cerebral cortical neurons. Compared to AAV vector treatment alone, behavioral and electrophysiological results showed that the combination therapy significantly improved upper limb motor functional recovery and neurotransmission efficiency after stroke. BDA tracing, immunofluorescence staining, qRT-PCR, and transmission electron microscopy of synaptic ultrastructure results revealed that the combination therapy not only potently increased the expression of Synapsin I, PSD-95, and GAP-43, but also promoted the axonal remodeling and restoration of abnormal synaptic structures. These findings provide a new strategy for enhancing neural plasticity and a potential means to treat stroke clinically.

1. Introduction

Stroke is the second leading cause of death globally, and leads to long-term disability in up to 50% of surviving patients (Donkor, 2018). Insufficient recovery of motor dysfunction largely affects the patient’s ability to live independently (Stinear, 2010). Except for recombinant tissue plasminogen activator reperfusion and surgical recanalization, which are severely limited by a narrow time window, no other effective therapies have yielded positive results in clinical trials to improve neuromotor impairment (Paul and Candelario-Jalil, 2021). Therefore, the development of new therapies to improve neuromotor outcomes after stroke is urgently needed.

Corticospinal tract (CST) is the long axon of motor neurons in the cerebral cortex and is the main transmission pathway of motor control from the sensorimotor cortex. CST axons transmit motor signals through spinal motor neurons and interneurons in the gray matter of the spinal cord and are the neuroanatomical basis of brain-controlled limb movements (Welniarz et al., 2017). After stroke, corticospinal innervation of the stroke-impaired side is disconnected by neuronal loss or axonal disruption, which leads to persistent synaptic loss, structural, and functional changes, leading to long-term disabilities in stroke patients (Hofmeijer and van Putten, 2012). During the recovery phase after...
stroke, the surviving neural network on the contralateral or ipsilateral side of the injury reorganizes in an attempt to generate new neural circuits to rewire the denervated spinal motor neurons (Gan et al., 2021; Kraft et al., 2018; Liu et al., 2015; Liu et al., 2013; Liu et al., 2007; Liu et al., 2008). Enhancing corticospinal synaptic connectivity may be an effective strategy to promote functional recovery after stroke.

Brain-derived neurotrophic factor (BDNF) is widely expressed in the neural tissues of the brain and spinal cord and is an important molecule involved in neuroplasticity. BDNF and its high-affinity receptor tropomyosin receptor kinase B (TrkB) mediate long-term potentiation (LTP), synaptic transmission, and regulate synaptic plasticity (Bergami et al., 2008; Camuso et al., 2022; Kang and Schuman, 1995; Lin et al., 2018). Despite past studies indicating BDNF/TrkB can exert therapeutic potential for stroke, current research primarily focuses on its regulation of synaptic plasticity of the hippocampal neurons, spinal cord, and promotes motor function recovery after intracerebral hemorrhage (Bergami et al., 2008; Camuso et al., 2022; Inoue et al., 2022; Kang and Schuman, 1995; Lee and Chao, 2001; Lin et al., 2018).

In this study, we investigated a non-invasive treatment method in rats utilizing intramuscularly injecting adenov-associated virus (AAV) vectors encoding BDNF into the stroke impaired forelimb muscles targeting the motor neurons in the anterior horn of the spinal cord, and intranasally administering AAV-TrkB targeting the cortical neurons and the axonal terminals of CST in the gray matter of the spinal cord. We found that overexpression of BDNF and TrkB in the spinal cord contralateral to the side of injury significantly promoted synaptic plasticity of the corticospinal connections and motor functional recovery after middle cerebral artery occlusion (MCAO). This provides a new strategy to enhance neural plasticity and a potential means of treating neurological diseases like strokes and other forms of neurological diseases.

2. Materials and methods

2.1. Animals and experimental design

A total of 174 adult male Sprague–Dawley (SD) rats (2 months old, 250–300 g) were used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shandong First Medical University and complied with guidelines outlined in the National Institute of Health’s Guide for the Care and Use of Laboratory Animals.

2.1.1. Experiment 1

For SD rats subjected to MCAO, AAV-GFP (1.71 × 10^13 vg/ml) or saline was injected intramuscularly into the left forelimb or intranasally 1 day after MCAO to assess transduction efficiency of the AAV vectors. Animalswere sacrificed on day 28 after MCAO. The cervical spinal cords of rats in the intramuscular injection group and the brains of rats in the intranasal administration group were processed to evaluate GFP expression.

2.1.2. Experiment 2

To demonstrate the therapeutic effect of BDNF-TrkB overexpression on neurological recovery and synaptic plasticity of the corticospinal connections in the denervated side of the spinal gray matter after stroke, animals were randomly divided into 5 groups: sham group (n = 22), MCAO-E group (MCAO surgery with AAV-empty intramuscular and intranasal injections, n = 33), MCAO-B group (MCAO surgery with AAV-BDNF vector intramuscular injection, 6.29 × 10^13 vg/ml, n = 32), MCAO-T group (MCAO surgery with AAV-TrkB intranasal injection, 4.71 × 10^12 vg/ml, n = 31), MCAO-BT group (MCAO surgery, AAV-BDNF intramuscular injection and AAV-TrkB intranasal injection, n = 38). Treatments were performed 1 day after surgery. Subsequent neurological assessments, electrophysiology, qRT-PCR, transmission electron microscopy, and all histological analyses were carried out in a double-blinded manner.

2.2. Ischemic stroke model

A permanent MCAO model was employed for ischemic stroke (Longa et al., 1989). Briefly, the right external carotid artery (ECA) in isoflurane-anesthetized rats was ligated distally and an MCAO-specific suture of 0.26 mm in length with a sintered hemispherical tip of 0.32–0.36 mm in diameter (Beijing Xinong Technology Co., Ltd., China) was advanced into the lumen of the internal carotid artery (ICA) through the proximal incision of the right ECA. In the sham group, the MCAO sutures were not inserted into the right ICA.

One day after the surgery, the Longa’s method was used for neurological deficit assessment, and the rats were scored on a four-point scale (0–4): 0) no deficit; 1) failure to fully extend left forepaw; 2) circling to the left; 3) falling toward the left; 4) no spontaneous walking with a depressed level of consciousness (Longa et al., 1989). Rats in the MCAO group with a score of 2 or 3 were included in the study and received further treatments with AAV vectors. The mortality rate of MCAO rats was 17.8% (n = 31) and the exclusion rate was 5.1% (n = 22).

2.3. AAV vector administration

All AAV vectors and related plasmids used in this study were constructed using serotype 2/ retrograde under the control of Syn promoter (Obio Technology, Shanghai, China).

2.3.1. Intranasal administration of AAV-vector

With minor modifications, AAV-GFP or AAV-TrkB was administered intranasally as previously described (Alcalí-Barraza et al., 2010). Ten 6-μl drops making up a full dose of 2 × 10^10 viral genomes of AAV-GFP or AAV-TrkB solution in saline were placed alternately into the right and left nostrils. The administration was performed at 3-min intervals between drops, allowing the rat to sniff naturally. The control group was given normal saline.

2.3.2. Intramuscular injection of AAV-vector

As previously described (Gan et al., 2021), under isoflurane anesthesia, 1 × 10^10 viral genomes of AAV-GFP or AAV-BDNF prepared in 10 μl saline per rat were injected into the wrist flexor muscles of the left forelimb. Injections were made into four points of 2.5 μl each. Animals in the control group were given 10 μl of saline.

2.4. Assessment of neurological function

To assess functional deficits and recovery, a single-pellet reaching test, a foot-fault test, and a von Frey test were performed. The rats were pretrained before surgery and tested weekly up to 8 weeks post-surgery. The single-pellet reaching test was performed to evaluate skilled hand function (Gan et al., 2021; García-Alias et al., 2015). Rats were placed individually in a transparent plastic box with a vertical groove on the front wall offering access to feed on the platform outside the box. Animals had to stretch their preferred forelimbs to reach and grasp the pellets. Each rat was allowed 20 grasps, and the ratio of the number of successfully acquired to the number of attempts was calculated.

The foot-fault test (Gan et al., 2021) was performed to evaluate nonskilled motor performance. Rats were placed on a non-equidistant steel frame with a grid at 1 m above the ground surface. Drops of the left forelimb paw from the grid were recorded as foot faults. A total of 50 steps were collected on the left forelimb. The percentage of foot faults was presented as faults/total steps × 100.

The von Frey test was used for quantitative measurement of the sensory threshold. Rats were placed on a wire mesh and acclimated for 30 min. A von Frey monofilament (ranging from 1 g to 26 g) was applied vertically to the left front paw, and withdrawal, licking, or retraction of the leg was defined as a positive response. Each filament was used five times and the paw withdrawal threshold (PWT) was calculated using a simplified up-down method with an adjustment factor of 0.5 (Bonin et al., 2014).
2.5. Electrophysiology

To examine the central motor pathway’s conduction, motor-evoked potential (MEP) was measured 8 weeks after MCAO. Electromyographic (EMG) recording was performed on the left forelimb carpi flexor muscle, which was evoked by electrical stimulation of the motor cortex surrounding the right stroke-injured brain. Animals were anesthetized and the right motor cortex was exposed. The stimulating electrodes were placed in the cortex sensorimotor area and the recording electrodes were inserted between the fascicles of the left forelimb carpi flexor, and the ground wire was placed under the tail skin. A square pulse stimulation inserted between the fascicles of the left forelimb carpi flexor, and the stimulation frequency of 4 Hz. EMG responses were recorded using an electromyographic device (Biopac mp150, USA), and latency period and wave amplitude were analyzed using Acknowledge software (Matsuda et al., 2020).

2.6. CST axon tracing

To detect changes in corticospinal connectivity, biotin dextran amine (BDA) (MW 10,000; 10% in PBS; molecular probe) was injected into five sites (coordinates: –1 mm anterior/1 mm lateral, –1 mm anterior/5 mm lateral, 4 mm anterior/1 mm lateral, 4 mm anterior/5 mm lateral, 3 mm anterior/2.5 mm lateral, depth: 1.5 mm) at 0.5 μl per site in the sensorimotor cortex contralateral to the injury 6 weeks after MCAO to trace CST axons. Animals were sacrificed 2 weeks post BDA injection. The BDA was visualized using streptavidin-cy3 (1:500, Solarbio, China). Corticospinal tract fibers at the cervical spinal cord (CS) were analyzed using denervated/intact side (%) as previously described (Ueno et al., 2012).

2.7. Immunofluorescence staining

Rats were transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA). The cervical cords or brains were removed, fixed in 4% PFA overnight, and transferred to PBS containing 30% sucrose formaldehyde (PFA). The cervical cords or brains were removed, fixed in 4% paraformaldehyde (PFA), and dehydrated with a gradient series of alcohol and acetone after being embedded 8 weeks after MCAO and transcardially perfused with cold PBS. Then, anterior horn of the spinal cord tissues were dissected, protected from light and fixed with 1% OsO4 in PBS for 2 h at room temperature and dehydrated with a gradient series of alcohol and acetone after being cut into 1-mm pieces. The samples were then resin infiltrated and embedded using acetone and EMBed 812 (SPI, USA). The resin blocks were cut into 70 nm slices and placed onto mesh cuprum grids, stained with 2% uranyl acetate saturated alcohol solution and 2.6% lead citrate and observed under an HT7800/HT7700 TEM (HITACHI, JAPAN). The synapse structures were examined as described before (Xu et al., 2009) and analyzed using ImageJ. Six images were examined per section in each group of four rats.

2.9. Transmission Electron microscopy (TEM)

The rats in the sham, MCAO-E, and MCAO-BT groups were anesthetized 8 weeks after MCAO and transcardially perfused with cold PBS. Then, anterior horn of the spinal cord tissues were dissected, protected from light and fixed with 1% OsO4 in PBS for 2 h at room temperature and dehydrated with a gradient series of alcohol and acetone after being cut into 1-mm pieces. The samples were then resin infiltrated and embedded using acetone and EMBed 812 (SPI, USA). The resin blocks were cut into 70 nm slices and placed onto mesh cuprum grids, stained with 2% uranyl acetate saturated alcohol solution and 2.6% lead citrate and observed under an HT7800/HT7700 TEM (HITACHI, JAPAN). The synapse structures were examined as described before (Xu et al., 2009) and analyzed using ImageJ. Six images were examined per section in each group of four rats.

2.10. Statistical analysis

All values were presented as mean ± SE. GraphPad Prism was used for statistical analysis and graph generation. Before statistical analysis, the Shapiro-Wilk (S–W) normality test and Bartlett test were applied to confirm normality and homogeneity of variance, respectively. Two-tailed Student’s t-test was used for comparison of differences between two groups, and one-way ANOVA with Dunnett’s post-hoc test was used for multiple comparisons. Two-way ANOVA with Bonferroni’s post hoc test was performed on neurological function data due to time and treatment factors. A value of P < 0.05 denotes statistical significance.

3. Results

3.1. AAV vectors are expressed in the sensorimotor cortical and spinal neurons after intranasal and intramuscular delivery

To validate the transport of AAV into the cerebral cortex after intranasal delivery in 5D rats subjected to MCAO, AAV-GFP was intranasally administered. Rats were sacrificed 4 weeks later. The brains were processed for immunocytochemistry and the transduction of AAV mediated gene was verified by colocalization of GFP with the neuronal nuclear marker (NeuN). As shown in Fig. 1 B–C, GFP was observed in the contralateral cerebral cortex to the injury and could be expressed in neurons in layer V. For the intramuscular injection, positive GFP expression was observed in approximately 31 neurons in the anterior horn of the spinal cord 4 weeks after administration, while no GFP positive cells were observed in the control group (Fig. 1 D–E). We also characterized the expression of TrkB in the cerebral cortex by immunofluorescence staining and detected the mRNA levels of BDNF and TrkB in the spinal cord 8 weeks after MCAO by qRT-PCR. Of the cortical neurons studied, the MCAO-T and MCAO-BT groups showed elevated immunostaining of TrkB than other groups (Fig. 2 A). Compared with the MCAO-E group, intramuscular injection of AAV-BDNF increased BDNF mRNA levels in the cervical enlargement of the spinal cord by 148% in the MCAO-B group (p < 0.01) and 158% in the MCAO-BT group (Fig. 2 B). In addition, intranasal administration of AAV-TrkB alone and AAV-TrkB combined with AAV-BDNF resulted in significant upregulation of TrkB expression levels in the cervical spinal cord where the corticospinal tract axons terminated. These results successfully demonstrated the AAV vector mediated gene expression by a neural specific Syn promoter in sensorimotor cortex and spinal cord neurons after intramuscular and intranasal administration.

J. Wang et al.  Experimental Neurology 359 (2023) 114236
3.2. Combination therapy with AAV-BDNF and AAV-TrkB promotes recovery of motor function after stroke

Staining of MAP-2 at 8 weeks post MCAO confirmed typical lesions in the cortex and striatum associated with stroke (Fig. 3B). The measured defect volume at 8 weeks post MCAO indicated treatment with the chosen AAV vectors did not change the infarct volume (Fig. 3C).

A series of neurological tests were performed to investigate whether AAV-BDNF or/and AAV-TrkB treatment could alleviate MCAO-induced long-term dyskinesia. All animals were trained before MCAO surgery, and the neurobehavior functions were assessed from week 1 up to 8 after surgery.

As shown in Fig. 3D, the MCAO-B, MCAO-T, and MCAO-BT groups showed statistically significant functional improvement from week 4 compared with the MCAO-E group. The success rate of single-pellet reaching increased earlier in the MCAO-B group compared with the MCAO-T group, and there was no significant difference between the two groups at 8 weeks after stroke. Notably, compared to AAV-BDNF and AAV-TrkB used alone, combination therapy resulted in the highest success rate among all treatment groups.

Similar to the skilled behavior, non-skilled motor behavior deficits evaluated using the foot fault-test were gradually but incompletely restored. As shown in Fig. 3E, compared with the MCAO-E group, treatment with AAV-BDNF or AAV-TrkB alone demonstrated a reduction in the foot fault rate from weeks 4 or 5, respectively. Combination treatment in the MCAO-BT group produced the best therapeutic effect among all the treatment groups.

In addition, the results of the mechanical pain threshold test showed that MCAO induced a 70% reduction of the withdrawal threshold with the left forepaw at week 1, followed by a gradual recovery. No significant differences among the four MCAO groups were observed throughout the experimental period (Fig. 3F). Taken together, these results suggest that combination therapy with AAV-BDNF and AAV-TrkB significantly promotes recovery of motor function, but has no significant effect on sensory function after stroke.
3.3. Combination therapy improves conduction of the central motor pathway

We combined MEPs with EMG to assess changes in cortical excitability and connectivity of central motor pathways 8 weeks after MCAO (Fig. 3A-C). The MCAO-E, MCAO-B, and MCAO-T groups had significantly longer latent period of EMG than the MCAO-BT group (Fig. 4C). These results indicate that electrophysiological conduction of the central motor pathway was better repaired in MCAO-BT group. Compared with the sham group, MCAO induced a reduction in the amplitude at week 8, and no significant differences in MEP amplitudes among the four treatment groups after MCAO were observed (Fig. 4D, p > 0.05). These results suggest that the combination therapy with AAV-BDNF and AAV-TrkB improves the central motor pathway’ conduction.

3.4. Combination treatment therapy upregulates the levels of multiple synaptic plasticity markers in the cervical spinal cord after stroke

To evaluate the synaptic plasticity after ischemic stroke, immunofluorescence staining was performed and the immunofluorescence density were measured to quantify the expression levels of presynaptic membrane protein Synapsin I, the postsynaptic density protein 95 (PSD-95), and growth-associated protein 43 (GAP43), which are plasticity markers involved in neurotransmission and synaptic plasticity and reorganization (Jovanovic et al., 1996; Kwon and Chapman, 2011; Lin et al., 2016; Migaud et al., 1998; Murphy and Corbett, 2009; Wu et al., 2004; Xia et al., 2017). MCAO induced downregulated expression of Synapsin I and PSD-95 compared with the sham group 8 weeks after MCAO, and this effect was significantly reversed by the combination treatment with AAV-BDNF and AAV-TrkB (Fig. 5A-D). For GAP-43, the sham group had very low expression levels compared to the other treatment groups. MCAO induced the upregulation of GAP-43, which was not significantly different from the MCAO-B and MCAO-T groups. Notably, combination therapy could significantly enhance GAP-43 levels in the cervical spinal cord compared to the other groups (Fig. 5E-F). These results suggest that combined treatment with AAV-BDNF and AAV-TrkB may promote neuronal axonogenesis and synaptic plasticity and the increase of BDNF and TrkB expression should be the key therapeutic mechanism of the combined treatment with AAV-BDNF and AAV-TrkB after cerebral ischemic.

To further support the findings identified by immunofluorescence staining, we analyzed the mRNA expression of each synapse-related protein in spinal cord. With minor exceptions, the mRNA expression levels of each protein closely mimicked fluorescence density (Fig. 5G-I).

3.5. Combined treatment improves synaptic formation in the anterior horn of the spinal cord after stroke

We assessed synaptic plasticity in the anterior horn of the spinal cord using TEM and presented representative images of the observed synaptic structures in Fig. 6A-C. Fig. 6A showed in the sham group, the presynaptic and postsynaptic membranes, the synaptic cleft, and the PSD can be seen in relatively clear outline. Eight weeks after MCAO the number of synapses were significantly reduced in the MCAO-E group (p < 0.01).
compared to the sham group, while the number of synapses (Fig. 6D) in the MCAO-BT group were significantly more than those in the MCAO-E group (p < 0.05). By analyzing the synaptic interface structure, we found that compared to the sham group, the thickness of PSD (Fig. 6E) in the MCAO-E group was significantly reduced and the width of the synaptic cleft (Fig. 6F) was increased. However, combination therapy within the MCAO-BT group alleviated the synaptic deficiencies caused by MCAO. In addition, the synaptic interface curvature was not significantly different among groups (Fig. 6G). These results suggest that combined treatment with AAV-BDNF and AAV-TrkB promotes repair of the synaptic structure after stroke (Fig. 8).

### 3.6. Combination therapy promotes CST axonal remodeling in the gray matter of the spinal cord

To determine whether intact CST axons sprouted into denervated spinal cord gray matter 8 weeks after MCAO, BDA was injected into the left cortex and the axons growing to the left across the midline in the gray matter of the spinal cord were assessed. In the sham group of rats, BDA-labeled fibers mainly terminated in the middle layer of the right spinal cord, with a few in the anterior horn of the spinal cord (Fig. 7A). Eight weeks after MCAO, BDA-labeled axons sprouting across the midline of the spinal cord toward the denervated side were increased in all four MCAO groups compared with the sham group. Combination treatment further significantly enhanced stroke-induced axonal reorganization in the spinal cord and the sprouted axons were able to reach the middle layer and the anterior horn of the spinal cord (Fig. 7B-F).
4. Discussion

Mediated by its receptor TrkB, BDNF plays a critical role in synaptic plasticity and transmission. Several methods that targeted delivery of neurotrophic factors to the central nervous system have been reported (Bahlakeh et al., 2021). In the present study, we developed a targeted delivery strategy to specifically overexpress BDNF/TrkB via AAV vectors in the denervated spinal gray matter in rats. AAV vectors were employed to deliver genes into motor neurons in the denervated spinal anterior horn by intramuscular injection, and neurons in the brain by intranasal delivery. Consistent with previous studies (Alcalà-Barraza et al., 2010; Gan et al., 2021; Segal, 2003; Thorne et al., 2004), our results showed that the AAV vectors were expressed in the motor neurons of the anterior horn of the spinal cord 4 weeks after intramuscular injection, and were expressed in the sensorimotor cortex and anterior horn of the spinal cord 4 weeks after intranasal administration. In the grey matter of the spinal cord, BDNF binds TrkB, thereby promoting corticospinal synaptic plasticity and strengthening synaptic connections to restore the corticospinal innervation. We determined that this combined delivery of BDNF/TrkB resulted in recovery of upper extremity motor function after MCAO, with long-term effects on synapse formation and corticospinal connectivity, rather than just transient plasticity.

In the present study, we estimated the neurological outcome of the stroke-impaired left forepaw using the foot-fault test and the single-pellet reaching test, which require voluntary control of the paw. Although motor function recovery was observed in rats over time, however, insufficient in the fine and skilled voluntary movement control detected as decreased grasp reaching and impaired grasp release of the hemiplegic limb. We found that the combination therapy significantly promoted upper limb voluntary motor function compared to delivering BDNF or TrkB alone, beyond the minimal spontaneous recovery found in the non-treated group in stroke rats. Interestingly, no long-term significant therapeutic effect was observed after exogenous BDNF or TrkB administration alone, which may require proper internalization and transport of the ligand-receptor complex (Bergeron et al., 1995; Pencea et al., 2001; Zhang et al., 2000).

BDNF/TrkB is also involved in pain regulation in the peripheral and central nervous systems. While our study focused on BDNF/TrkB delivery specifically targeting the denervated side of the spinal anterior horn, off-target effects may result from this anatomically targeted expression strategy, as TrkB also reaches the cerebral cortex, brain stem, and other parts of the cervical spinal cord (Belur et al., 2017; Thorne et al., 2004; Wolf et al., 2012). Increased TrkB abundance within these aforementioned regions may lead to axonal outgrowth of sensory neurons, thereby inducing allodynia and hyperalgesia. Our results showed that treatment did not alter nerve injury-induced allodynia and hyperalgesia in rats after MCAO, as assessed by mechanical pain threshold testing. This may be because TrkB requires more BDNF to modulate pain perception at off-target sites, which requires a balance between the pro-nociceptive and anti-nociceptive effects of BDNF/TrkB (Nijs et al., 2015).
Fig. 6. Synaptic ultrastructure detected using TEM. (A-C) Representative images in the (A) sham group, (B) MCAO-E group, and (C) MCAO-BT group. (D-G) Quantification of the (D) number of synapses, (E) width of the synaptic cleft, (F) thickness of postsynaptic density and (G) the curvature of synaptic interface. *p < 0.05, **p < 0.01, ns vs. the MCAO-E group, n = 4/group, scale bar = 1 μm, ns = not significant.

Fig. 7. BDA axon labeling in the spinal cord. (A-E) Representative images of BDA-positive labeling in the spinal cord of experiment groups 8 weeks after MCAO. (F) Quantitative analysis of BDA-positive nerve fibers in the spinal cord (n = 4/group). ***p < 0.01 vs. the MCAO-E group, ### p < 0.01 vs MCAO-BT group, scale bar = 200 μm.
Mediated by the receptor tyrosine kinase TrkB, BDNF regulates synaptic transmission and long-term potentiation (LTP) by inducing phosphorylation of synaptic proteins or altering synaptic protein composition by local protein synthesis (Bathina and Das, 2015; Chen et al., 2017; Lv et al., 2018). The application of BDNF has been described to enhance synaptic transmission through presynaptic (Lohof et al., 1993) or postsynaptic mechanisms (Levine et al., 1995). BDNF binds to TrkB and induces phosphorylation of its downstream effector Synapsin I, a protein found on small synaptic vesicles, which is closely involved in synapse formation and remodeling, through the mitogen-activated protein kinase (MAPK) cascade in cortical neurons and PC12 cells (Jovanovic et al., 1996; Kwon and Chapman, 2011; Wu et al., 2004; Xia et al., 2017). PSD is the structural basis of postsynaptic plasticity. As the most abundant postsynaptic scaffolding protein, PSD-95 regulates the structure of synaptic junctions and transduces membrane receptor signaling by interacting with a variety of proteins (Lin et al., 2016; Migaud et al., 1998). In this study, combined treatment simultaneously increased the expression levels of BDNF and TrkB in the spinal cord, thereby increasing the expression of pre-and post-synaptic proteins (Synapsin I and PSD-95, respectively). Meanwhile, the levels of GAP-43 in the MCAO-BT group, which is only expressed in the nervous system and plays an important role in neural development, synaptic function, and neural regeneration (Chung et al., 2020; Murphy and Corbett, 2009), were higher compared to other treatment groups. In addition, the ultrastructure of synapses revealed by TEM showed that the relevant synaptic morphology indicators were altered by the combined treatment with AAV-BDNF and AAV-TrkB. These results indicate that expression of BDNF/TrkB and induces phosphorylation of its downstream effector Synapsin I, which is closely involved in synapse formation and remodeling. Additionally, BDNF enhances synaptic transmission which is related to the activation of TrkB receptors and depends on the location of the nervous system, the intrinsic properties of different neurons, the time point of delivery and the age of the subject (Hernandez-Echeagaray, 2020; Yan et al., 1992). This may also promote neurological recovery after stroke. Thus, studies on CST axonal tracing as well as causal loss experiments are warranted to further demonstrate the underlying mechanisms of BDNF/TrkB treatment promoting CST axonal sprouting and remodeling.

MEPs are used to assess the function of descending motor pathway and neurological dysfunction by stimulating the motor cortex (Maeda et al., 2021; Oinuma et al., 2007), which can provide information on the patients following stroke (Bakker et al., 2019; Hordacre et al., 2020). This present study showed no improvement in MEP amplitude, but a reduction latency, which seems to suggest that combination therapy may help restore neural conduction (Barker et al., 2012). However, due to the contribution of the spinal cord and supraspinal pathways, we cannot identify the source of functional connectivity enhancement of neural pathways and evaluate the pyramidal tract, so more precise electrophysiological methods may be required to assess other mechanisms, such as enhanced synaptic transmission.

Notably, a study using a transient MCAO rat model failed to demonstrate a significant difference in the number of CST terminals in the denervated side of the spinal cord originating from the intact brain hemisphere after MCAO, compared with that in sham-operated group (Mitchell et al., 2016). This seems to contradict our experimental results and other publications (Liu et al., 2013). The authors mention that differences in infarct location and severity may be due to the different methods and durations of arterial occlusion in different stroke models. Indeed, extensive reorganization of corticospinal connections is associated with axonal sprouting, development of new synapses, and increased myelination, and is important for motor recovery after stroke (Liu et al., 2008; Liu et al., 2009). An increase in structural connectivity of both ipsilateral and contralateral corticospinal pathways after ischemic

Fig. 8. Schematic diagram of the treatment strategy. I.M, intramuscular injection; I.N, intranasal administration; PSD-95, postsynaptic density-95; GAP-43, growth-associated protein-43.
stroke injury has been demonstrated in the past using DTI-based FA measures (Zolkefley et al., 2021), while its specific contribution to neural recovery remains unclear and needs to be further confirmed.

Taken together, findings of this work indicate treatment options that overexpress BDNF and its receptor TrkB in the spinal cord by intramuscular injection and intranasal administration support further studies for stroke treatment. While this study was aimed at the upper extremity motor function, we speculate similar approaches are applicable for the functional recovery of lower extremities. The present study focused on the effects on synaptic plasticity and reorganization, and future investigations focusing on other aspects of stroke-related effects, such as axonal sprouting and dendritic branching are warranted.

5. Conclusion

The present study shows that the combination of intramuscular administration of AAV-BDNF and intranasal administration of AAV-TrkB significantly enhances behavioral outcomes and increases synaptic plasticity and reorganization in the denervated gray matter of the spinal cord after ischemic stroke in rats. This provides a novel strategy for the treatment of stroke patients and may provide ideas for the treatment of other central nervous system diseases.

Data availability statement

The original datasets generated for this study are available from the corresponding author upon reasonable request.

Authors’ contributions

B.S., M.Y and L.M. designed the experiments. J.W. and Y.C. performed most of the experiments and statistical analyses. J.W. wrote the manuscript and L.M. and B.S. revised the manuscript with input from all authors. J.S., H.F., X.Z., Q.C, F.G. and Q.N helped to perform experiments.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported in part by grants of the Natural Science Foundation of China (No.81871855 to Y-MF and No. 81870938 to S-BL), Natural Science Foundation of Shandong Province (No.ZR2019DZ32 to S-BL, No. ZR2021HQ160 to S-JY, No.ZR2021MH025 to N-QB), Ji’nan Science and Technology Development Project (No. 20201916 to S-JY), by Fund of Taishan Scholar Project and Fund of Academic Promotion Program of Shandong First Medical University & Shandong Academy of Medical Sciences (No.2019QL016).

References

Alcaí-Barraza, S.R., Lee, M.S., Hanson, L.R., McDonald, A.A., Frey 2nd, W.H., McLean, L.K., 2010. Intranasal delivery of neurotrophic factors into the central nervous system: focus on available approaches. Cell Biosci 11, 181.

Bakker, C.D., Massa, M., Daffertshofer, A., Pasman, J.W., van Kuijk, A.A., Kwakkel, G., Stegeman, D.F., 2019. The addition of the MEP amplitude of finger extension muscles to clinical predictors of hand function after stroke: a prospective cohort study. Restor. Neurol. Neurosci. 37, 445–456.

Barker, R.N., Brauer, S.G., Barry, B.K., Gill, T.J., Carson, R.G., 2012. Training-induced modifications of corticostriatal reactivity in severely affected stroke survivors. Exp. Brain Res. 221, 211–223.

Bathina, S., Das, U.N., 2015. Brain-derived neurotrophic factor and its clinical implications. Arch. Med. Sci. 11, 1164–1178.

Belur, L.R., Temme, A., Podsedek, K.M., Rindl, M., Volchnova, L., Robinson, N., Hamond, L.R., Kozaryn, K.F., Orchard, P.J., Frey 2nd, W.H., Low, W.C., McVor, R.S., 2017. Intranasal adeno-associated virus mediated gene delivery and expression of human-iduronidase in the central nervous system: a noninvasive and effective approach for prevention of neurological disease in mucopolysaccharidosis type I. Hum. Gene Ther. 28, 576–587.

Bergami, M., Rimondini, R., Santì, S., Blum, R., Goitz, M., Canossa, M., 2008. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc. Natl. Acad. Sci. U. S. A. 105, 15570–15575.

Bergeron, J.J., Di Guglielmo, G.M., Baas, P.C., Authier, F., Fonser, B.I., 1995. Endosomes, receptor tyrosine kinase internalization and signal transduction. Biosci. Rep. 15, 411–418.

Bonin, R.P., Borjes, C., De Koninck, Y., 2014. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol. Pain 10, 26.

Boroneant, G.A., Strick, P.L., 1993. Corticospinal terminations in two new-world primates: further evidence that corticomotorneuronal connections provide part of the neural substrate for manual dexterity. J. Neurosci. 13, 5105–5118.

Camuso, S., La Rosa, P., Fiorenza, M.T., Canterini, S., 2022. Pleiotropic effects of BDNF on the cerebellum and hippocampus: implications for neurodevelopmental disorders. Neuropsychiol. Dis. 163, 105506.

Chen, X., Wang, X., Tang, L., Wang, J., Shen, C., Liu, J., Lu, S., Zhang, H., Kuang, Y., Fei, J., Wang, Z., 2017. Nhe5 deficiency enhances learning and memory via upregulating Bdnf/Ttrk signaling in mice. Am. J. Med. Genet. B Neuropsychiatr. Genet. 174, 828–838.

Chung, D., Shum, A., Caraveo, G., 2020. Gap-43 and Basp1 in axon regeneration: implications for the treatment of neurodegenerative diseases. Front Cell Dev Biol 8, Article 567537.

Donkor, E.S., 2018. Stroke in the 21(st) century: a snapshot of the burden, epidemiology, and quality of life. Stroke Res Treat 2018, 3281615.

Durick, D.A., Huston, T.H., Kathe, C., Solomon, S., Gonzalez-Carter, D., Petruska, J.C., Shine, H.D., Chen, Q., Wood, T.C., Bernanos, M., Cash, D., Williams, S.C., Gage, F.H., Moon, L.D., 2016. Delayed intramuscular human neurotrophin-3 improves recovery in adult and elderly rats after stroke. Brain 139, 259–275.

Fortun, J., Puzis, R., Perone, D.D., Gage, F.H., Bunge, M.R., 2009. Muscle injection of AAV-NF3 promotes anatomical reorganization of CST axons and improves behavioral outcome following SCI. J. Neurotrauma 26, 941–953.

Gan, X., Chopp, M., Xin, H., Wang, F., Golembieski, W., Lu, M., He, L., Liu, Z., 2021. Targeted CRT overexpression in denervated spinal motor neurons promotes stroke recovery in mice. J. Cereb. Blood Flow Metab. 41, 92–104.

Garcia-alias, G., Truong, K., Shah, P.K., Roy, R.R., Edgerton, V.R., 2015. Plasticity of subcortical pathways promote recovery of skilled hand function in rats after corticospinal and rubrospinal tract injuries. Exp. Neurol. 266, 112–119.

Hernandez-Echeagaray, E., 2020. The role of the TrkB-T1 receptor in the neurotrophin-4/5 antagonism of brain-derived neurotrophic factor on corticostriatal synaptic transmission. Neural Regen. Res. 15, 1973–1976.

Hofmeijer, J., van Putten, M.I., 2012. Ischemic cerebral damage: an appraisal of synaptic failure. Stroke 43, 607–615.

Hordacre, B., Goldsworthy, M.R., Welby, E., Graetz, L., Ballinger, S., Hillier, S., 2020. Resting state functional connectivity is associated with motor pathway integrity and upper-limb behavior in chronic stroke. Neurorehabil. Neural Repair 34, 547–557.

Inoue, T., Takamatsu, Y., Nishio, T., Soma, K., Okamura, M., Tohyama, H., Maqimi, H., 2022. Combined treatment with exercise and dGABA(A)/R inhibitor promotes motor function recovery after intracerebral hemorrhage. Neurosci. Lett. 766, 136344.

Imazilov, A.A., Povyeshova, T.V., Bashirov, V.V., Sokolov, M.E., Fadeev, P.O., Garfulin, R.R., Naroitsky, B.S., Logunov, D.Y., Salafutdinov, I.I., Chelyshev, Y.A., Islamov, R.R., Lavrov, I.A., 2017. Spinal cord cellular and molecular changes induced by adenosivector and cell-mediated triple gene therapy after severe contusion. Front. Pharmacol. 8, 813.

Jiang, Y.Q., Armada, K., Martin, J.H., 2019. Neuronal activity and microglial activation support corticospinal tract and proprioceptive afferent sprouting in spinal circuits after a corticospinal system lesion. Exp. Neurol. 321, 113015.

Kraft, A.W., Bauer, A.Q., Calver, J.P., Lee, J.M., 2018. Sensory deprivation after focal ischemia in mice accelerates brain remodeling and improves functional recovery toward arc-dependent synaptic plasticity. Sci. Transl. Med. 10, 445ra121.

Kwon, S.E., Chapman, E.R., 2011. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 70, 847–854.

Lee, K.H., Chao, M.V., 2001. Activation of TrkB neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. U. S. A. 98, 3555–3560.
Lee, J.K., Kim, J.E., Sivala, M., Strittmatter, S.M., 2004. Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. J. Neurosci. 24, 6292–6217.

Lemon, R.N., 2008. Descending pathways in motor control. Annu. Rev. Neurosci. 31, 195–218.

Levine, E.S., Dreyfus, C.F., Black, I.B., Plummer, M.R., 1995. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc. Natl. Acad. Sci. U. S. A. 92, 8074–8077.

Lin, H., Jacoby, A.A., Anderson, S.A., Lynch, D.R., 2016. D-serine and serine racemase are associated with PSD-95 and glutamatergic synapse stability. Front. Cell. Neurosci. 10, 34.

Lin, P.Y., Kavali, E.T., Monteggia, L.M., 2018. Genetic dissection of presynaptic and postsynaptic BDNF-TrkB signalling in synaptic efficacy of CaA-CaA synapses. Cell Rep. 24, 1550–1561.

Liu, Z., Li, Y., Qu, R., Shen, L., Gao, Q., Zhang, X., Lu, M., Savant-Bhonsale, S., Bornehm, J., Chopp, M., 2007. Axonal sprouting into the denervated spinal cord and synaptic and postsynaptic protein expression in the spinal cord after transplantation of bone marrow stromal cell in stroke rats. Brain Res. 1149, 172–180.

Liu, Z., Li, Y., Zhang, X., Savant-Bhonsale, S., Chopp, M., 2008. Contralesional axonal remodeling of the corticospinal system in adult rats after stroke and bone marrow stromal cell treatment. Stroke 39, 2571–2577.

Liu, Z., Zhang, R.L., Li, Y., Cui, Y., Chopp, M., 2009. Remodeling of the corticospinal innervation and spontaneous behavioral recovery after ischemic stroke in adult mice. Stroke 40, 2546–2551.

Liu, Z., Chopp, M., Ding, X., Cui, Y., Li, Y., 2013. Axonal remodeling of the corticospinal tract in the spinal cord contributes to voluntary motor recovery after stroke in adult mice. Stroke 44, 1951–1956.

Liu, G., Dang, C., Chen, X., Xing, S., Dami, K., Xie, C., Peng, K., Zhang, J., Li, J., Zhang, J., Chen, L., Pei, Z., Zeng, J., 2015. Structural remodeling of white matter in the contralesional hemisphere is correlated with early motor recovery in patients with subcortical infarction. Restor. Neurol. Neurosci. 33, 309–319.

Liu, Y., Wang, X., Li, W., Zhang, Q., Li, Y., Zhang, Z., Zhu, J., Chen, B., Williams, P.R., Zhang, Y., Yu, B., Gu, X., He, Z., 2017. A sensitized GFG treatment restores corticospinal axon-dependent functions. Neurogn 95, 817–833.e814.

Lohof, A.M., Ip, N.Y., Poo, M.M., 1993. Potentiation of developing neuromuscular synapses by the neurotrophin NT-3 and BDNF. Nature 363, 350–353.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion in the rat: A new model of aneurismal subarachnoid hemorrhage and cerebral ischemia. Stroke 20, 8–18.

Lv, C., Ma, Q., Han, B., Li, J., Geng, Y., Zhang, X., Wang, M., 2018. Long-term DL-3-n butylphthalide treatment alleviates cognitive impairment correlated with improving synaptic plasticity in SAMP8 mice. Front. Aging Neurosci. 10, 200.

Lv, H., Li, Y., Cheng, Q., Chen, J., Chen, W., 2021. Neuroprotective effects against cerebral ischemic injury exerted by Dexamethasone via the HDAC5/NPAS4/MDM2/PSD-95 Axis. Mol. Neurobiol. 58, 1990–1996.

Maeda, Y., Oinuma, T., Mitsuhara, T., Okazaki, T., Yuge, L., Takeda, M., 2021. A novel bone-thinning technique for transcranial stimulation motor-evoked potentials in mice. J. Neurosci. 41, 1232–1237.

Matsuda, M., Kanno, H., Sugaya, T., Yamaya, S., Yahata, K., Handa, K., Shindo, T., Shimokawa, H., Ozawa, H., Itoi, E., 2020. Low-energy extracorporeal shock wave therapy promotes BDNF expression and improves functional recovery after spinal cord injury in rats. Exp. Neurol. 328, 113251.

Mckay, M., Charlesworth, P.P., Dempster, M., Webster, L.C., Watabe, A.M., Makhlison, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., O’Dell, T.J., Grant, S.G., 1998. Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396, 433–439.

Mitchell, E.J., Dewar, D., Maxwell, D.J., 2016. Is Remodelling of corticospinal tract terminations originating in the intact hemisphere associated with recovery following transient ischemic stroke in the rat? PLoS One 11, e0152176.

Murabe, N., Mori, T., Fukuda, S., Isou, N., Ohno, T., Mizakami, H., Ozawa, K., Yoshimura, Y., Sakurai, M., 2018. Higher primate-like direct corticomotorneural connections are transiently formed in a juvenile subprimate mammal. Sci. Rep. 8, 16536.

Murphy, T.H., Corbitt, D., 2009. Plasticity during stroke recovery: from synapse to behaviour. Nat. Rev. Neurosci. 10, 861–872.

Nijj, J., Mees, M., Versijpt, J., Moors, M., Bos, J., Knoepen, K., Meersen, R., 2015. Brain-derived neurotrophic factor as a driving force behind neuroplasticity in neuropathic and central sensitization pain: a new therapeutic target? Expert Opin. Ther. Targets 19, 565–576.

Ohimana, M., Suzuki, K., Honda, T., Matsumoto, M., Sasaki, T., Kodama, N., 2007. High-frequency monopolar electrical stimulation of the rat cerebral cortex. Neurosurgery 60, 189–196 (discussion 196–197).

Paul, S., Candelario-Jalil, E., 2021. Emerging neuroprotective strategies for the treatment of ischemic stroke: an overview of clinical and preclinical studies. Exp. Neurol. 335, 113518.

Pencea, V., Bingaman, K.D., Wiegand, S.J., Lukin, M.B., 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J. Neurosci. 21, 6706–6717.

Petruka, J.C., Kitay, B., Boyce, V.S., Kaspar, B.K., Pearse, D.D., Gage, F.H., Mendell, J.M., Corbett, D., 2009. Intramuscular AAV delivery of NT-3 alters synaptic transmission to motoneurons in adult rats. Eur. J. Neurosci. 32, 997–1005.

Rostlon, D.D., Ralston 3rd, H.J., 1985. The terminations of corticospinal tract axons in the macaque monkey. J. Comp. Neurol. 242, 325–337.

Segal, R.A., 2003. Selectivity in neurotrophin signaling: theme and variations. Annu. Rev. Neurosci. 26, 299–330.

Stinear, C., 2010. Prediction of recovery of motor function after stroke. Lancet Neurol. 9, 1228–1232.

Thorne, R.G., Prok, G.J., Padmanabhan, V., Frey 2nd, W.H., 2004. Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. Neuroscience 127, 481–496.

Ueno, M., Hayano, Y., Nakagawa, H., Yasumori, T., 2012. Intraspinal rewiring of the corticospinal tract requires target-derived brain-derived neurotrophic factor and compensates lost function after brain injury. Brain 135, 1253–1267.

Welniaq, Q., Dusart, I., Rose, E., 2017. The corticospinal tract: evolution, development, and human disorders. Dev Neurobiol 77, 810–829.

Wolf, D.A., Hanson, L.R., Aronovich, E.L., Nan, J., Low, W.C., Frey 2nd, W.H., McVor, R.S., 2012. Lysosomal enzyme can bypass the blood-brain barrier and reach the CNS following intranasal administration. Mol. Genet. Metab. 106, 131–134.

Wu, A., Ying, Z., Gomez-Pinilla, F., 2004. Dietary omega-3 fatty acids normalize BDNF levels, reduce oxidative damage, and counteract learning disability after traumatic brain injury in rats. J. Neurotrauma 21, 1457–1467.

Xia, W.G., Cheng, C.J., Zhang, X., Wang, J., 2017. Effects of ‘nourishing liver and kidney’ acupuncture therapy on expression of brain derived neurotrophic factor and synaptophysin after cerebral ischemia in rats. J. Huazhong Univ Sci Technolog Med Sci 37, 271–278.

Xu, X., Ye, L., Ruan, Q., 2009. Environmental enrichment induces synaptic structural modification after transient focal cerebral ischemia in rats. Exp Biol Med (Maywood) 234, 296–305.

Yan, Q., Elliott, J., Snider, W.D., 1992. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. Nature 360, 753–755.

Zhang, Y., Yu, B., Gu, X., He, Z., 2017. A sensitized GFG treatment restores corticospinal axon-dependent functions. Neurogn 95, 817–833.e814.