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Effect of fluoxetine on HIF-1α- Netrin/VEGF cascade, angiogenesis and neuroprotection in a rat model of transient middle cerebral artery occlusion

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Running head The influence of fluoxetine on ischemic rat brain

Abstract

Fluoxetine is one of the most promising drugs for improving clinical outcome in patients with ischemic stroke. This in vivo study investigated the hypothesis that fluoxetine may affect HIF-1α-Netrin/VEGF cascade, angiogenesis and neuroprotection using a rat model of transient middle cerebral artery occlusion (tMCAO). The rats were given fluoxetine or saline after tMCAO for 4 weeks. Then, protein expression of HIF-1α-Netrin/VEGF cascade was examined at 1, 2, 4 weeks after tMCAO. In vivo synchrotron radiation were performed to observe microangiography of ischemic brain after 4 weeks of tMCAO. The infarct size and neurobehavioral test were carried out 1 to 4 weeks after tMCAO. Results revealed that HIF-1α expression was upregulated in fluoxetine-treated group. Similarly, fluoxetine increased protein expression of Netrin and its receptor DCC, VEGF and its receptor VEGFR. Synchrotron radiation angiography revealed more branches in fluoxetine-treated rats. We found no difference of infarct volume between fluoxetine and saline treated rats after 1 week of tMCAO, and ischemia-induced brain atrophy volume in fluoxetine-treated group was attenuated after 4 weeks of tMCAO. Neurological deficits were improved in fluoxetine-treated rats at 3 and 4 weeks after tMCAO. Our results indicated that fluoxetine could upregulate protein expression of HIF-1α-Netrin/VEGF cascade, promote angiogenesis, and improve long-term functional recovery after ischemic stroke.

Keywords Fluoxetine, Middle cerebral artery occlusion, Hypoxia-inducible factor-1α, Netrin-1, Vascular endothelial growth factor

1. Introduction

Ischemic stroke is the major cause of disability and second most common cause of death worldwide. In the acute stage of ischemic stroke, recombinant tissue plasminogen activator (rt-PA) and mechanical thromboectomy are still preferred treatment. Due to the limitation of time windows of recanalization therapy, the majority of patients have to seek medication and rehabilitation therapy. But none of the medicines was proved to be effective on the improvement of functional recovery after acute ischemic stroke.

Several studies showed that selective serotonin reuptake inhibitors (SSRIs) might improve clinical outcome in patients with cerebral infarction, particularly promote motor function recovery (Chollet et al., 2011; Loubinoux et al., 2002; Robinson et al., 2000). Fluoxetine for Motor Recovery after Acute Ischemic Stroke (FLAME) study indicated that the motor scale of fluoxetine group was improved significantly compared with the control group 3 months after index stroke (Chollet et al., 2011). But recently, the FOCUS trial (Fluoxetine or Control Under Supervision) did not identify the superiority of fluoxetine administration to alter the modified Rankin Scale at 6 months after the onset of stroke over placebo (Collaboration, 2019). Most importantly, the effect of SSRIs on ischemic stroke is still not clear in animal models and the mechanisms may include the following aspects. First, several studies have shown that fluoxetine may exert the neuroprotective role against the immune inflammatory response via the repression of microglia activation, neutrophil infiltration, and pro-inflammatory marker expressions (Lim et al., 2009; Zhang et al., 2012). Second, fluoxetine may act on calcium channel of the vascular smooth muscle and dilate the arterioles, thereby increasing blood supply in brain and ischemic penumbra (Bonne et al., 1996; Ofek et al., 2012; Ungvari et al., 1999). Third, previous in vivo studies suggested that chronic fluoxetine treatment could attenuate the cognitive

deficits through the increasing hippocampal neurogenesis after ischemic stroke (Li et al., 2009; Ohira and Miyakawa, 2011). In addition, our previous in vitro study indicated that fluoxetine might induce VEGF/Netrin hyperexpression via the mediation of hypoxia-inducible factor 1-alpha (HIF-1α) in SH-SY5Y cells (Wang et al., 2016). Therefore, in this in vivo study we aimed to test the hypothesis that fluoxetine may exert a positive effect on ischemic brain through HIF-1α-Netrin/VEGF cascade using a rat model of transient middle cerebral artery occlusion (tMCAO). After tMCAO we investigated expression of HIF-1α, Netrin-1/VEGF and their receptors, angiogenesis, infarct size and long-term functional outcome.

2. Materials and methods

2.1. Animals and the experimental design

All experimental animal procedures followed the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), and the guidelines of the regulation for the administration of affairs concerning experimental animals of China enacted in 1988. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the School of Medicine, Shanghai Jiao Tong University, Shanghai, China. All rats were housed under standard laboratory conditions. Adult male Sprague-Dawley rats (Harlan, RRID:RGD_10395233) weighting 250-300g aged eight to ten weeks were used in this study. Male rats were used in the present study due to the neuroprotective effect of sex hormones in females. Animals were housed 2 or 3 to a cage with ad libitum access to chow and water, under temperature-controlled conditions on a 12 hour (h) light/dark cycle. All rats were randomly divided into each group using the GraphPad software randomization tool (http://www.graphpad.com/quickcalcs/randomize1.cfm). Eighty-one rats used to assess the protein

level after 1, 2, 4 weeks of tMCAO were divided into three groups: (1) fluoxetine-treated group, (2) saline-treated group, (3) sham-operated group. Sixteen rats used to evaluate the angiogenesis detected by Proliferating Cell Nuclear Antigen (PCNA) / CD31 staining after 4 weeks of tMCAO were divided into two groups: (1) fluoxetine-treated group, (2) saline-treated group. Twenty-four rats used to test branches of microvessels by live synchrotron radiation angiography after 4 weeks of tMCAO were divided into three groups (1) fluoxetine-treated group, (2) saline-treated group, (3) sham-operated group. Thirty-two rats used to evaluate the infarct volume at 1 and 4 weeks after tMCAO were divided into two groups: (1) fluoxetine-treated group, (2) saline-treated group. Twenty-four rats used to test neurobehavioral outcome 1, 2, 3, 4 weeks after tMCAO were divided into three groups: (1) fluoxetine-treated group, (2) saline-treated group, (3) sham-operated group. The investigators, performing the evaluation of outcomes (molecular biology analyses, synchrotron radiation microangiography, infarct size, behavioral test), were blinded to treatment groups (coded animals, samples). The experimental design is summarized in Fig. 1.

2.2. Transient middle cerebral artery occlusion model

The operation procedure of the transient middle cerebral artery occlusion (tMCAO) has been previously described (Lin et al., 2013). Rats were deeply anesthetized with 2% isoflurane (Maingrette et al., 2015) and were immediately placed on a thermoregulated heating pad (RWD Life Science, Shenzhen, China). Body temperature was maintained at 37±0.5°C throughout the surgical procedure. The absence of response to toe pinch indicated deep anesthesia, and the right common carotid artery (CCA), external and internal carotid arteries (ECA and ICA) were isolated. A monofilament nylon filament with a diameter of 0.26 mm and a length of 40 mm (Beijing Cinontech Biotech Co. Ltd, Beijing, China) coated with silicon hardener mixture (a diameter of 0.34 ± 0.02 mm) was inserted into

the ECA stump, then reversed into the ICA and finally to the ostium of the right middle cerebral artery (MCA). The success of occlusion was characterized by the reduction in MCA cerebral blood flow down to 20% of the baseline, which was monitored by a laser Doppler flowmetry (Moor Lab, Instruments, Axminister, UK). In fluoxetine- and saline-treated groups, MCA occlusion was maintained for 2 hour before the suture was gently withdrawn from the artery to allow reperfusion. The local blood flow was evaluated again by the laser Doppler flowmetry after the suture withdrawn, and an increase of cerebral blood flow to 70% of the baseline was verified as successful reperfusion. Those rats with hemiparesis appeared after more than 1 hour of occlusion, indicating successful tMCAO, were included in the experiment. The success rate of tMCAO model was 75%. The unsuccessful modeling rats were discarded and the mortality of rats underwent tMCAO were 15%. In sham-operated group the operation was done in the same way except for the filament insertion. In fluoxetine-treated group, fluoxetine hydrochloride (Eli Lilly, France) was dissolved in 0.89% NaCl and delivered by oral gavage (20 mg/kg/day) once a day for 4 weeks after 24 hours of tMCAO; this was a dose proved to reach serum levels of fluoxetine that are equivalent to therapeutic doses used to humans (Dulawa et al., 2004; David et al., 2009; Maingrette et al., 2015). In saline-treated and sham-operated groups, equivalent 0.89% NaCl was administered. Rats were sacrificed at predetermined time points after surgery using an overdose of sodium pentobarbital (500 mg/kg intraperitoneal, Provet, Malaga, WA, Australia).

2.3. Assessment of HIF-1α-Netrin/VEGF cascade protein levels after tMCAO by western blotting

Proteins of brain samples of right hemispheres were loaded on 11% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis at 110V for 90 min. Subsequently, proteins were transblotted onto polyvinylidene difluoride membranes by the wet transfer (400 mA, 90 min). The membrane was

placed in Tris-buffered saline Tween-20 (TBST) containing 5% nonfat milk for 2 hours at 25°C and incubated with the following primary antibodies: mouse monoclonal antibody against HIF-1α (1:400, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal antibody against Netrin-1 (1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal antibody against UNC5H2 (1:800, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal antibody against DCC (1:400, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse monoclonal antibody against VEGFA (1:500, Santa Cruz Biotechnology, Inc., Dallas), mouse monoclonal antibody against VEGFR-2 (1:400, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. After washing with TBST, the blots were incubated with the secondary antibody (mouse monoclonal antibody against Beta-Actin, 1:4000, Santa Cruz Biotechnology, Santa Cruz, TX, USA) for 2 hours at room temperature and then reacted with enhanced chemiluminescence substrates (Pierce, Rockford, IL, USA). The result of chemiluminescence was recorded and analyzed with image processing software (Image J, version 1.46, National Institutes of Health (NIH), Bethesda, MA, USA): relative contents of proteins were calculated by dividing the optical density of the target band with the optical density of β-actin band.

2.4. Immunofluorescence

Brain sections were blocked with 10% bovine serum albumin following incubation with goat polyclonal antibody against CD31 (1:300, R and D systems, Minneapolis, MN, USA) and mouse monoclonal antibody against PCNA (1:1000, Abacm, Cambridge, England) overnight at 4°C. Sections were then incubated with fluorescence-conjugated secondary antibodies. Each experiment had appropriate positive and negative controls for each batch of slides. The number of PCNA/CD31-positive cells was assessed at 400× in 3 microscopic fields at the left, right, and bottom of peri-infarct area.

2.5. Synchrotron radiation microangiography in vivo

Synchrotron radiation microangiography was conducted at the BL13W beamline of Shanghai Synchrotron Radiation Facility (SSRF) at 4 weeks after tMCAO. After anesthesia with 2% isoflurane, tMCAO rats were immediately placed on a heating pad (37°C) during the imaging process. A PE-10 tube connected with a syringe pump (LSP01-1A, Longer pump; Baoding, China) was carefully inserted into the proximal CCA. The rat was placed vertically to the X-ray beam on its right side. X-ray energy was 33.2 keV, just above the iodine K-edge energy. Non-ionic iodine contrast agent (150μL, 350mg/mL, Omnipaque, GE, Fairfield, CO) was injected from CCA into ICA through the angiographic tube at a rate of 133.3μl/s. A PCO x-ray charge-coupled device (CCD) camera (PCO-TECH Inc, Germany) was placed 650 mm from the sample stage. Two layers of dynamic images were acquired to obtain the entire hemisphere vasculature every 172 ms (Lin et al., 2015).

2.6. Infarct volume measurement

Animals were reperfused by 0.89% NaCl and 4% paraformaldehyde and frozen immediately at 1 and 4 weeks after tMCAO. A series of 20-μm-thick coronal slices from anterior commissure to hippocampus were cut and mounted on slides, then stained by cresyl violet. The measurement of infarct and atrophy volume was performed by a person who is blind to the treatment history of the samples. Slices were imaged and digitized, and infarct volume was calculated in mm^3 using Image J (version 1.46, National Institutes of Health (NIH), Bethesda, MA, USA). Brain atrophy was calculated by subtracting the volume of intact tissue in the ipsilateral hemisphere from that of the contralateral hemisphere .

2.7. Behavioral tests

Animals were trained and assessed for 3 days before surgery. Baseline values were generated

simultaneously by averaging 3 trials, and rats were tested once a week at 1, 2, 3, 4 weeks after tMCAO between 9 and 11 am. The beam-walking test was used to measure the ability of motor coordination and balance. Rats traversed a horizontally elevated square beam with 2.4 cm wide and 80 cm long beam to reach an enclosed safety platform. The scoring method varying from 0 to 6 allocated for balanced with a steady posture (Liu et al., 2009). Motor test data were analyzed as mean score to keep balance on the beam from three trials. The ladder rung walking task allows assessing skilled walking and measuring both forelimb and hindlimb placing, stepping and inter-limb coordination. Rats were placed on a horizontal ladder 14 cm wide and 1 meter long rung walkway with irregularly at 2-5 cm intervals (between rungs), suspended 30 cm above the surface. Rats were video-recorded as they traversed the ladder 3 times. Three trials were evaluated and presented as an average performance per group. The method and scoring system ranged from 0 (abnormal) to 6 (perfect) according to video recordings (Metz and Whishaw, 2002, 2009). All data of error scores and errors per step were presented as percent of 100% values of the respective control groups as defined by the following formula: (average of lesion animals/average of controls)×100.

2.8. Statistics

Statistical analyses were performed with Prism 7.0d (GraphPad Software Inc., San Diego, CA, USA). All data were presented as mean ± standard deviation (SD) of at least three independent determinations in the text and figures and a statistical difference was accepted at less than 5% level with 95% confidence intervals. Statistical analysis of data that passed either tests for normality were performed with an unpaired t - test for comparisons between two groups or one - or two -way analysis of variance (ANOVA) with Tukey-Kramer or Sidak's post hoc test for multiple group comparisons. Kruskal-Wallis test with Dunnett post hoc test was used for nonparametric data.

3. Results

3.1. Effect of fluxetine on expression of HIF-1α-Netrin/VEGF cascade in a rat transient middle cerebral artery occlusion model

We first established a rat tMCAO model and treated the model with fluoxetine for 4 weeks. Western blot analysis showed that HIF-1α expression was upregulated in the ipsilateral hemispheres of tMCAO in fluoxetine-treated group compared with other groups after 2 and 4 weeks of ischemia (Fig. 2A). Fig. 2B unveiled that fluoxetine increased HIF-1α expression with statistical significance between fluoxetine and saline treated group after 2 and 4 weeks of tMCAO ($p<0.01$).

Based on literature research and our results of in vitro study, we found that fluoxetine may increase Netrin expression. We decided to explore whether fluoxetine induces expression of Netrin and its receptors in vivo. First, we constructed a rat tMCAO model and treated the model with saline or fluoxetine. Our results showed that in saline-treated group, Netrin expression increased in the ischemic hemispheres at 2 weeks after tMCAO compared with sham group (Fig. 2A and 2C, $p<0.01$), indicating that in focal ischemia, Netrin expression was enhanced, and fluoxetine further upregulated Netrin expression after 1, 2, 4 weeks compared with saline group (Fig. 2A and 2C, $p<0.01$ or $p<0.05$). Second, we tested expression of Netrin receptors of UNC5H2 and DCC. We found that in saline-treated group, UNC5H2 expression was increased in the ischemic hemispheres after 1, 2, 4 weeks compared with sham group (Fig. 2A and 2D, $p<0.01$), but fluoxetine did not upregulate UNC5H2 after 1 and 2 weeks and decreased UNC5H2 expression after 4 weeks compared with saline-treated group (Fig. 2A and 2D, $p<0.01$). Furthermore, the results from protein measurement showed that in saline-treated group, focal ischemia increased DCC after 2 and 4 weeks compared with sham group (Fig. 2A

and 2E, $p<0.01$), and fluoxetine further upregulated DCC expression in the ischemic hemispheres after 2 and 4 weeks of tMCAO compared with saline-treated group (Fig. 2A and 2E, $p<0.01$ and $p<0.05$).

To detect the effect of fluoxetine on VEGF, we measured protein expression of VEGF in the ischemic hemispheres of saline or fluoxetine-treated groups. Western blotting showed that VEGF expression was significantly upregulated in the ischemic hemispheres in fluoxetine-treated rats compared with saline-treated rats after 2 and 4 weeks of tMCAO (Fig. 2A and 2F, $p<0.01$). Similarly, expression of VEGF receptor protein was increased in fluoxetine-treated group at 4 weeks after tMCAO compared with saline-treated group (Fig. 2A and 2G, $p<0.01$), and in saline-treated rats VEGF receptor was also upregulated at 1 and 2 weeks compared with sham group (Fig. 2A and 2G, $p<0.01$).

3.2. Effect of fluoxetine on angiogenesis in a rat transient middle cerebral artery occlusion model

We used PCNA and CD31 dual-labeled immunofluorescence to detect proliferation of vascular endothelial cells in peri-infarct area at 4 weeks after tMCAO. We found that PCNA-positive cells expressed CD31, indicating the presence of proliferating endothelial cells. Significantly more PCNA/CD31-positive cells were observed in fluoxetine-treated rats compared with saline-treated rats (Fig. 3A and 3B, 6.99 ± 0.98 vs 2.29 ± 0.39 cells/field, $p<0.001$).

Furthermore, we observed branches of MCA in living animals using synchrotron radiation angiography. We found branches of right MCA (ischemic side) in fluoxetine-treated rats were greater than that in saline-treated rats at 4 weeks after tMCAO, suggesting that fluoxetine could promote local angiogenesis after focal ischemia (Fig. 3C). The number of MCA branches within the left side of tMCAO rats or the sham group was consistent. However, quantification of vessel branches using synchrotron radiation angiography is still a challenge same as in CT and MR angiography. Software to quantify vessel branches based on synchrotron radiation angiography, is still being developed.

3.3. Effect of fluoxetine on infarct size after stroke

We then examined whether fluoxetine affected the outcome after focal ischemia. Unstained volume in the ipsilateral hemispheres of tMCAO was measured to assess infarct size after 1 and 4 weeks of tMCAO. As shown in Fig. 4, fluoxetine administration resulted in a unremarkable reduction of infarct volume compared with saline-treated rats at one week after tMCAO (64.13±5.13 vs 69.39±4.81, $p = 0.053$). At 4 weeks after tMCAO, brain atrophy volume was significantly reduced in fluoxetine-treated rats compared with saline-treated rats (Fig. 4, 68.54±5.91 vs 82.35±5.54, $p<0.01$), suggesting that fluoxetine could improve histological outcome after focal ischemia.

3.4. Effect of fluoxetine on Neurological outcomes

To assess whether fluoxetine affected the neurological deficits after focal ischemia, neurobehavioral outcome was measured after 1- 4 weeks of focal ischemia. There was no significant difference in performance of beam-walking test and the ladder rung walking task among sham, fluoxetine-, and saline-treated groups before tMCAO. However, neurological deficits were significantly improved in fluoxetine-treated rats than in saline-treated rats at 3 and 4 weeks after tMCAO (Fig. 5, $p<0.05$ or $p<0.01$).

Discussion

For ischemic stroke, current research on the mechanism of fluoxetine including the following aspects: improvement of post-stroke depression (Robinson et al., 2000), enhancement of blood supply of ischemic areas (Bonne et al., 1996; Ungvari et al., 1999), neuroprotection in ischemic brain tissues (Lim et al., 2009), neural remodeling and revascularization (Zhang et al., 2012). Previously, we hypothesized that fluoxetine may affect the proteins involved in neural remodeling and

revascularization, and we confirmed that fluoxetine induced expression of Netrin and VEGF via mediation of HIF-1α under hypoxia in SH-SY5Y cells (Wang et al., 2016). In the present study, we demonstrated that fluoxetine upregulated HIF-1α-Netrin/VEGF cascade, promoted angiogenesis, and improved neurological outcome in a rat transient middle cerebral artery occlusion model.

HIF-1α is a key transcription regulator for multiple angiogenic factors including Netrin-1 and VEGF under hypoxia (Pugh and Ratcliffe, 2003; Rosenberger et al., 2009; Tsuzuki et al., 2000). It can regulate gene expression, angiogenesis and other biological effects for systemic, tissue and cellular adaption to hypoxia. In this study, we found that fluoxetine did not affect HIF-1α expression after one week of ischemia, but increased HIF-1α after 2 and 4 weeks. Accordingly, in our previous study we proved fluoxetine did not change the level of HIF-1α protein in vitro after hypoxic treatment for 24 h, and fluoxetine upregulated Netrin and VEGF expression via mediation of HIF-1a that binds to hypoxia response element (HRE) (Wang et al., 2016). It is believed that HIF-1α has positive and negative effects on ischemic brain tissue. On the one hand, HIF-1α could induce a series of downstream target genes such as erythropoietin, VEGF, Netrins, inducible nitric oxide synthase, which may promote neuro- and vascular regeneration (Semenza, 2014). On the other hand, HIF-1α may take part in blood-brain barrier disruption and apoptosis via the cytokines neuroinflammatory reaction to hypoxia in glial cells (Higashida et al., 2011; Koh et al., 2015; Yeh et al., 2011). It has been proved that HIF prolyl hydroxylase inhibition prior to transient focal cerebral ischemia is neuroprotective in mice and selective inhibition of expressed HIF-1α may be beneficial at the early- but not late- stage of hypoxia in rat after focal ischemic brain damage (Yeh et al., 2011). It is thus obvious that the influence of HIF-1α on ischemic infarction is complicated, which seems closely related to the time after hypoxia, and fluoxetine may provide neuroprotection effect via upregulation of HIF-1α after 1 week of hypoxia

in tMCAO rat model.

The study showed that fluoxetine could induce Netrin-1 and VEGF expression after ischemia in tMCAO rats. Formation of vascular network shares some similarities with neural system. Netrin-1 and VEGF are potent mitogens that regulate angiogenesis through multiple ways, including the stimulation of endothelial cell proliferation, migration and tube formation (Fan et al., 2008; Ferrara et al., 2003; Park et al., 2004; Wilson et al., 2006). It has been established that overexpression of Netrin-1 and VEGF can induce neovascularization, reduce infarct size and improve motor function recovery in the animal model of cerebral ischemia (Lu et al., 2012; Sun et al., 2011; Sun et al., 2003; Yang et al., 2017). Previously, in a mouse model of tMCAO we found that adeno-associated viral vectors carrying Netrin-1 gene injected into the brain can induce Netrin-1 hyperexpression, promote neovascularization, reduce infarct size and improve functional recovery (Lu et al., 2012). Netrin can attract or repel axonal growth through interaction with its receptors DCC (Deleted in Colorectal Cancer) and UNC5 (uncoordinated-5) family members (Hong et al., 1999), but the role of DCC and UNC5 in Netrin-induced angiogenesis is still under debate (Lu et al., 2004; Navankasattusas et al., 2008; Nguyen and Cai, 2006). We found that fluoxetine promoted the expression of DCC after 2 and 4 weeks of ischemia, presumably via a DCC-dependent ERK1/2-eNOS feed-forward mechanism to induce angiogenesis (Nguyen and Cai, 2006). VEGF-A binds to VEGF receptor-2 (VEGFR2) and is the most important biological signaling of VEGF mediated cerebral angiogenesis. VEGFR2 inhibition may promote endothelial cell death and limit cell proliferation in a neonatal rodent model of stroke (Shimotake et al., 2010). Fluoxetine has been shown to induce endothelial cell proliferation in the subgranular zone via VEGF-FIK-1 signaling (Warner-Schmidt and Duman, 2007) and restore ischemia-induced neovascularization significantly in mice exposed to psychological stress (Maingrette et al., 2015). In

this study, we found that fluoxetine promoted protein expression of HIF-1α, Netrin-1, VEGF and their receptors, and was related to angiogenesis in pari-infarct area in tMCAO rat model. Based on our previous in vitro study, that indicating fluoxetine upregulated Netrin and VEGF expression via mediation of HIF-1α (Wang et al., 2016), we suggest that fluoxetine may present neuroprotective effect by promoting the HIF-1α- Netrin/VEGF cascade.

The possible mechanisms of fluoxetine related augmentation of motor function have been reported as follows: (1) promotion of neurogenesis (Khodanovich et al., 2018; Li et al., 2009; Maya Vetencourt et al., 2008); (2) anti-inflammation effect in the postischemic brain (Lim et al., 2009); (3) induction of vasodilation (Ofek et al., 2012); (4) reestablishment of inhibitory neural network tonus (Pinto et al., 2017); (5) upregulation of allopregnanolone in female rat brain (Fry et al., 2014). Steroidal hormones, viz. progesterone, estrogens, and testosterone play a role in neuroprotection and other brain injuries (Siddiqui et al., 2016). In this study, male rats were used to establish the tMCAO model, which limited the possibility to explore the neuroprotective effect of fluoxetine in female animals. Our study shows that fluoxetine may improve motor function during convalescence stage of stroke in a tMCAO rat model, which can be explained partially by the neovascularization effect via upregulation of HIF-1α-Netrin/VEGF cascade. However, the current report establishes a preliminary association between the HIF-1α-Netrin/VEGF cascade and fluoxetine-mediated neuroprotection. Further experiments modulating the activities of HIF-1α-Netrin/VEGF cascade are needed to confirm the results.

In conclusion, our findings shows that fluoxetine is related positively to neovascularization in ischemic brain of rats, probably via activating the HIF-1α-Netrin/VEGF cascade, which may contribute to long-term functional outcome. We hope this study will lay a theoretic basis of fluoxetine

administration on ischemic stroke therapy, and further studies are needed to provide more information on molecular mechanisms of fluoxetine improving cerebral infarction recovery.

Ethics approval

Rats experiments were approved by the Institutional Animal Care and Use Committee of the School of Medicine, Shanghai Jiao Tong University, Shanghai.

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Authors' contributions

Conception and design of study: QW, QH, XC, LL, LZ; Acquisition of data: LL, LZ, HL, QH, JW, XC; Animal model: LL, LZ; Western blot and immunofluorescence: QH, HL; Synchrotron radiation microangiography, infarction assessment and neurobehavioral test: JW, XC; Analysis and interpretation of data: QH, QW; Wrote the manuscript: QH, LZ, LL, QW.

Declaration of Competing Interests

The authors have no competing financial interest to declare.

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Fig. 1. Experimental design and schedules

tMCAO = transient middle cerebral artery occlusion, HIF-1α = hypoxia inducible factor-1α, vascular endothelial growth factor = VEGF

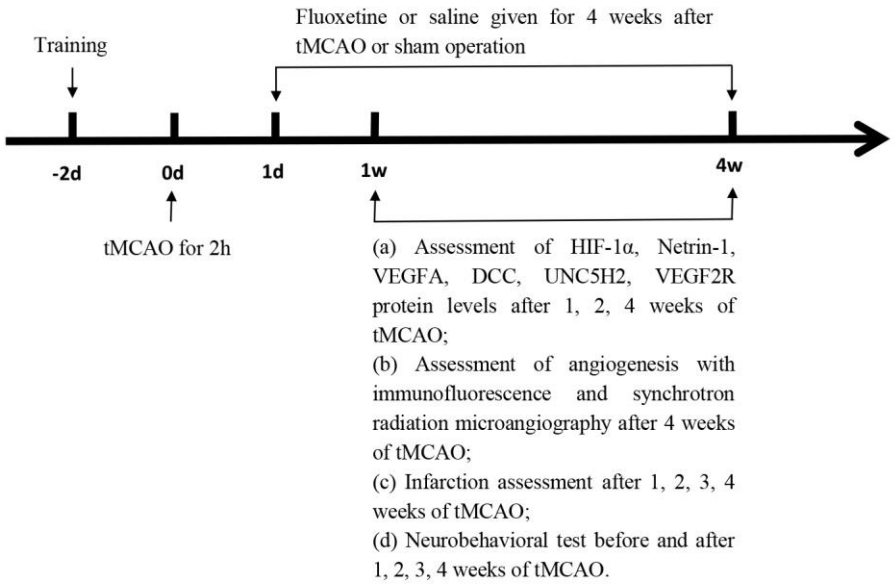
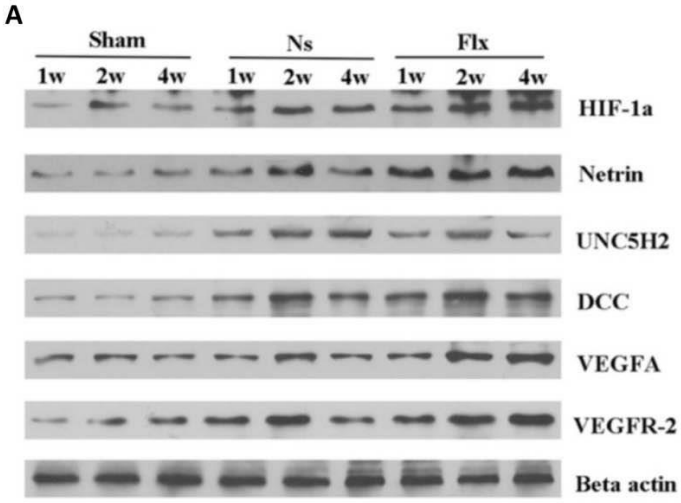
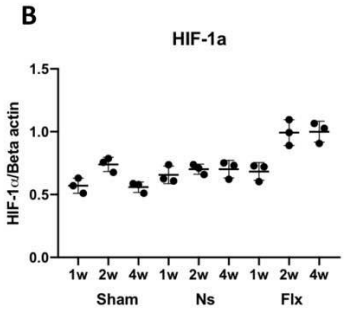


Fig. 2. Effect of fluoxetine on HIF-1α-Netrin/VEGF cascade in transient middle cerebral artery occlusion (tMCAO) rat model.

A. Protein expression of HIF-1α, Netrin and its receptor DCC/UNC5H2, VEGFA and its receptor VEGFR2 in the ischemic hemispheres after 1, 2, 4 weeks of tMCAO.

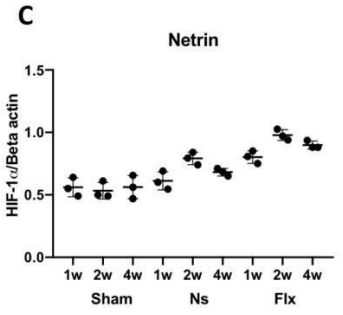
B-G. The optical density of the target band divided by the optical density of the beta-actin band. Data are expressed as mean±SD of at least three independent experiments. n=8 animals per group. w = week, Ns = saline, Flx = fluoxetine





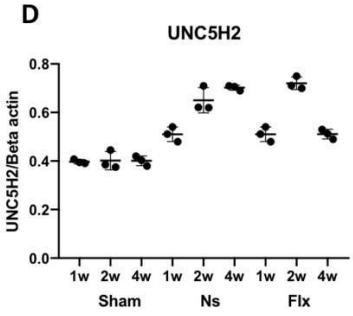
Tukey-Kramer test

	P value
2w Ns vs Flx	0.0015
4w Ns vs Flx	0.0012



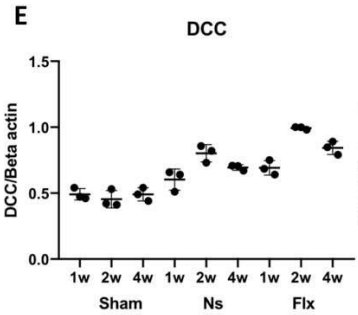
Tukey-Kramer test

	P value
1w Ns vs Flx	0.0249
2w Sham vs Ns	0.0014
2w Ns vs Flx	0.0285
4w Ns vs Flx	0.0081



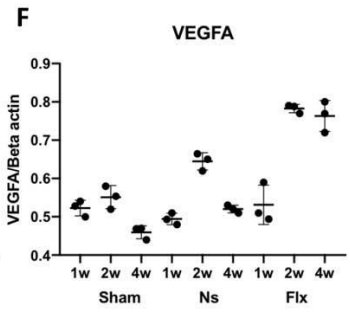
Tukey-Kramer test

	P value
1w Sham vs Ns	0.0038
2w Sham vs Ns	< 0.0001
4w Sham vs Ns	< 0.0001
4w Ns vs Flx	< 0.0001



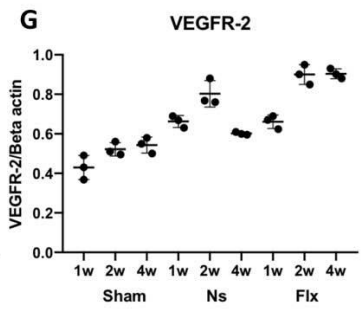
Tukey-Kramer test

	P value
2w Sham vs Ns	< 0.0001
2w Ns vs Flx	0.0072
4w Sham vs Ns	0.0038
4w Ns vs Flx	0.0396



Tukey-Kramer test

	P value
2w Sham vs Ns	0.0135
2w Ns vs Flx	0.0002
4w Ns vs Flx	< 0.0001



Tukey-Kramer test

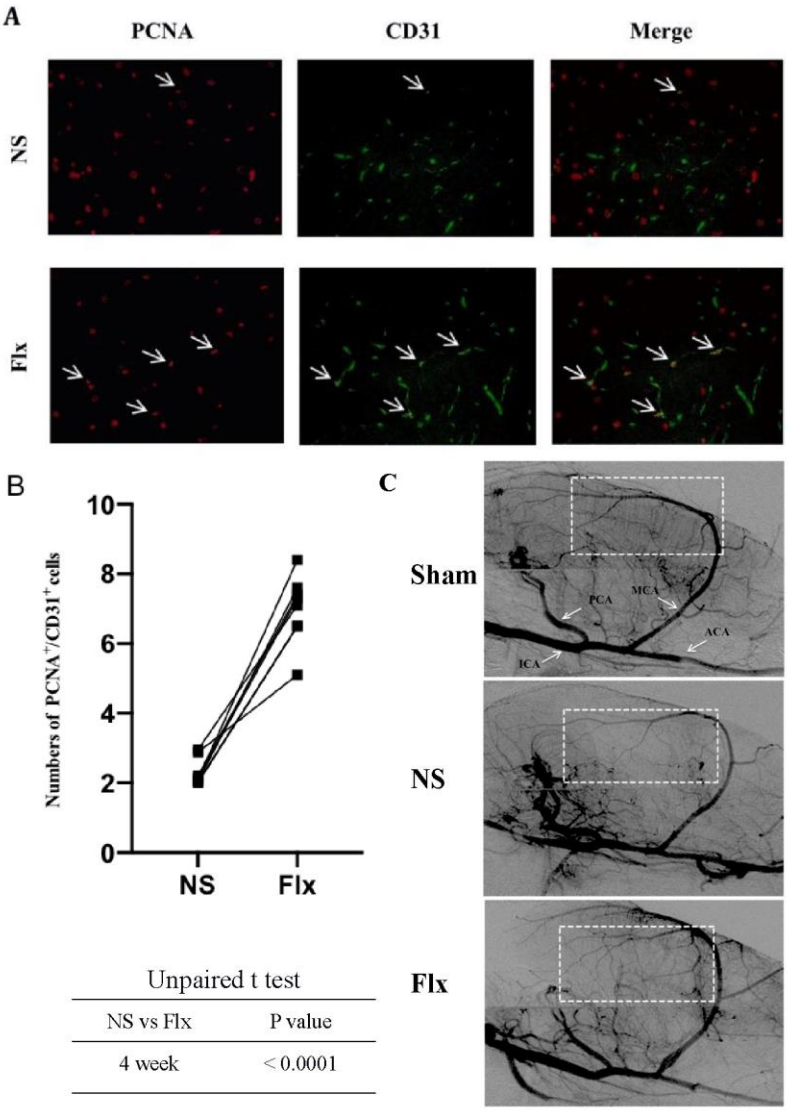
	P value
1w Sham vs Ns	< 0.0001
2w Sham vs Ns	< 0.0001
4w Ns vs Flx	< 0.0001

Fig. 3. Fluoxetine promoted endothelial cells proliferation in transient middle cerebral artery occlusion (tMCAO) rat model.

A. Representative images of PCNA-positive (red) cells and CD31-positive (green) cells in ischemic penumbra of rats treated with saline (NS) and fluoxetine (Flx) at 4 weeks after tMCAO. Bar=50 μm.

B. Quantification of PCNA/CD31-positive cells in the penumbra of rats at 4 weeks after treatment of saline (NS) and fluoxetine (Flx). n=8 animals per group.

C. Live synchrotron radiation angiography revealed newly formed vessels in tMCAO rats treated with fluoxetine. Photomicrographs show the perfusion of the right middle cerebral artery (MCA) territory in sham-operated, saline (NS)- and fluoxetine (Flx)- treated groups at 4 weeks after tMCAO. MCA branches perfused by contrast media are illustrated in the square. ICA, PCA, ACA indicate internal carotid artery, posterior cerebral artery and anterior cerebral artery.

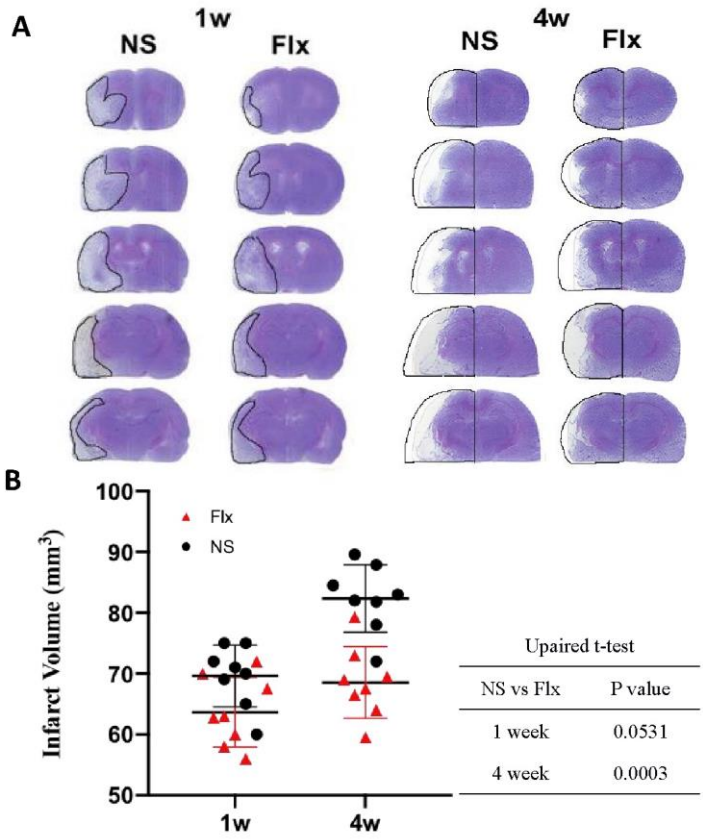


Unpaired t test	
NS vs Flx	P value
4 week	< 0.0001

Fig. 4. Cerebral infarct volume and brain atrophy after transient middle cerebral artery occlusion (tMCAO).

A. The brain infarct volume and brain atrophy was detected by cresyl violet staining in coronal brain sections treated with saline (NS) or fluoxetine (Flx) at 1 and 4 weeks after tMCAO. The line presented brain infarction at one week and the inversing contralateral that had no ischemia at 4 weeks. Brain atrophy was calculated by subtracting the volume of intact tissue in the ipsilateral hemisphere from that of the contralateral hemisphere.

B. Quantification of the infarct volume and brain atrophy. n=8 in each group.



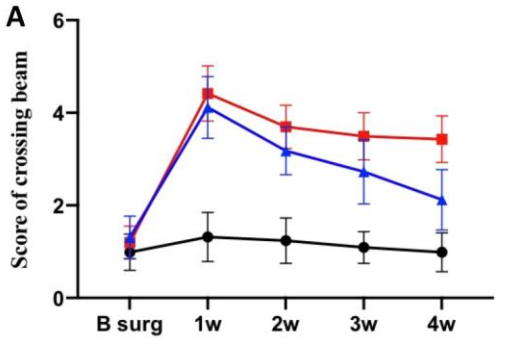
Upaired t-test	
NS vs Flx	P value
1 week	0.0531
4 week	0.0003

Fig. 5. Effect of fluoxetine on neurobehavioral outcome in transient middle cerebral artery occlusion (tMCAO) rat model.

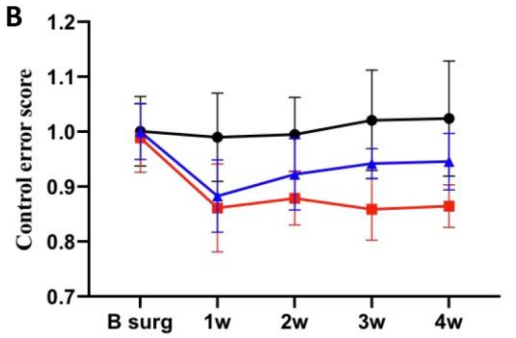
For neurobehavioral testing, rats were trained before tMCAO and neurobehavioral deficits were assessed by beam-walking and ladder rung walking tests in rats treated with sham, saline (NS) and fluoxetine (Flx) 1, 2, 3, 4 weeks after tMCAO. n=8 per group.

A. Neurological deficits were evaluated by beam-walking test.

B. Neurological deficits were evaluated by ladder rung walking test. B surg = before surgical procedure of tMCAO.



Tukey-Kramer test	
NS vs Flx	P value
1 week	0.4705
2 week	0.1190
3 week	0.0102
4 week	0.0001



Tukey-Kramer test	
NS vs Flx	P value
1 week	0.7924
2 week	0.3892
3 week	0.0371
4 week	0.0432