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Original Article

Fluoxetine alleviates cerebral ischemic injury by regulation of Notch1 signaling pathway in rats

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Abstract: Cerebral ischemic disease is a severe disease that is detrimental to human health, the purpose of the study was to determine the protective role of fluoxetine and roles of Notch1 signaling pathway in adult ischemic rats. In our study, a total of forty-eight male Wistar rats were randomized divided into 4 groups: The sham-operated group (Group A), the MCAO group (Group B), the MCAO + fluoxetine group (Group C), and the MCAO + fluoxetine + DAPT (Notch 1 signal pathway inhibitor) group (Group D). Zea Longa score was performed to evaluate the nervous response on days 1, 14 and 28 after the operation. After the last behavioral assessment, the rats were anaesthetized and the brain tissue was harvested to detect the expression of NICD, Hes1, Hes5, Jag1 mRNA with Real Time-PCR, soft tissue samples extracted from the brain tissue were lysed to analyze the expression of NICD, Hes1, Hes5, Jag1. Alternatively, the rats were perfused with 4% paraformaldehyde to evaluate NOTCH1 with immunohistochemistry in the brain. We found that Middle cerebral artery was occlusion (MCAO) could severely spoil the nervous functions; administration of fluoxetine alleviated the nervous damages significantly, while DAPT aggravated the motor function damage. Thecerebral ischemia due to MCAO decreased the NICD, Hes1, Hes5, Jag1 expression on both mRNA and protein level in brain tissue, combined with reduced Notch1 protein level, fluoxetine treatment upregulated the NICD, Hes1, Hes5, Jag1 mRNA level, and enhanced the concentration of NICD, Hes1, Hes5, Jag1 and Notch1 in brain tissue compared with the MCAO group, while DAPT downregulated the NICD, Hes1, Hes5, Jag1 mRNA level, combined with reduced concentration of NICD, Hes1, Hes5, Jag1 and Notch1 compared with the fluoxetine group. In summary, fluoxetine treatment can improve the motor function damage caused by cerebral ischemia; the underlying mechanism for the protective role might be modulation of Notch1 signaling pathway.

Keywords: Cerebral ischemia disease, fluoxetine, DAPT, Notch1 signaling pathway

Introduction

Cerebral infarction is a severe disease that can impair motion and cognition functions, which may also destroy the patients' quality of life and bring heavy burden for the family and the society, although thrombolytic therapy with intravenous tissue plasminogen activator (tPA) is an effective method for the treatment of acute cerebral infarction in patients, however, the rigid therapeutic time window has severely restricted its clinical application, a large number of patients have to undergo the acutest stage treatment [1]. Therefore, it's urgent to discover novel mechanisms and methods to alleviate the serious consequences.

The pathogenesis of cerebral ischemic injury is a complex process and the former studies mainly focused on the cellular ionic balance, energy metabolism, the inflammatory factors and oxidative stress. In recently years, the roles of cell signaling pathway in cerebral ischemic injury and the repair mechanism are increasingly valued, of all the related pathways, the study of the MAPK pathway, the PI3K-Akt pathway, and the Notch1 pathway have attracted more attentions [2].

The Notch signaling pathway is a highly conserved cell signaling system present in most eukaryocytes, and it involves multifacet biological processes including neuronal function and development, cell cycle transition etc. The Notch signaling pathway composed of the ligands (Jagged1, Jagged2, Delta1, Deha3 and Delta4), the receptors (Notch1, Notch2, Notch3, Notch4) and the downstream effectors, upon

the binding of ligands to receptors, Notch signaling pathway is activated and the intracellular domain Notch (NICD) is released and therefore induces the transcription of Notch target genes (Hes1, Hes5etc) [3]. Redmond et al [4] has demonstrated that Notch1 signaling exerts an important regulatory influence on the specification of dendritic morphology in neurons in vitro, Presente et al [5] reported that Notch protein persistently expressed in aging adult Drosophila brains and Notch activity is constitutively required in the adult fly for neurological function. Cheng et al [6] showed that Notch and HIF-1α collaborate in the activation of apoptotic, pro-inflammatory, and neurodegenerative pathways during brain injury following ischemia-reperfusion, which demonstrated that Notch signaling pathway might play an important role in the cerebral ischemic injury.

Fluoxetine is a selective serotonin reuptake inhibitor drug that is commonly used to treat depression, in addition to its anti-post stroke depression effect, Dam et al [7] suggested that it also facilitate recovery in post stroke patients undergoing rehabilitation. However, its pharmacologic actions on cerebral ischemia disease are still uncertain, for example, Jolkkonen et al [8] showed that subchronic fluoxetine treatment following experimental focal cerebral ischemia is not effective for sensorimotor recovery or attenuate spatial learning deficits for rats subjected to transient focal cerebral ischemia.

In the present study, we aim to study the functions of fluoxetine after the surgery of MCAO in rats, in addition, the role of Notch1 signal pathway were also examined to explore the underlying mechanisms in the pathogenesis of cerebral ischemic injury.

Materials and methods

Animals and grouping

Forty-eight male Wistar rats weighed 180-220 g were used in the study. The animals were provided by Shanghai SJA Laboratory Animal Co, Ltd in China. The rats were individually housed in a temperature-controlled room with a 12 h light/dark cycle and can be free access to water and food. This study was performed with the approval of the Board of Ethics of Jinshan Branch of Shanghai Sixth People's Hospital. All experimental procedures in this study were approved by the rules of our institutional animal care and use committee. After acclimation for 2 weeks, the rats were randomly divided into four groups (n = 12 per group). The rats were treated as follows: Group A, The sham-operated group, in which no middle cerebral artery was occluded; For the remaining three groups, rats were subjected to the surgery of MCAO followed by intraperitoneally injection of sterile saline 2 mg/kg on a daily basis (Group B); fluoxetine 2 mg/kg, intraperitoneally on a daily basis (Group C); and fluoxetine 2 mg/kg plus DAPT 5 mg/kg intraperitoneally on a daily basis (Group D).

Drugs and preparations

The fluoxetine hydrochloride (CAS Number 56296-78-7), DMSO (CAS Number 67-68-5), DAPT (CAS Number 208255-80-5), antibodies such as anti-β-actin (A5441) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-NICD (Sc-6014), anti-Hes1 (Sc-166410), anti-Hes5 (Sc-13859) and anti-Jag1 (Sc-8303) were from Santa Cruz, USA. The anti-Notch1 (#3608) was acquired from Cell Signaling Technology, Inc, USA, while the anti-α-SMA Antibody (catalog NO. 252037) was purchased from Abbiotec. Fluoxetine was diluted with sterile physiological saline (pH 7.4) to a final concentration of 1 mg/ml; DAPT was dissolved in DMSO as the vehicle.

Animal procedures

The Wistar rat cerebral infarction model was established as follows: rats were anesthetized with intraperitoneally 10% chloral hydrate (250 mg/kg), then the rats were placed in the supine-position and the body temperature was continuously monitored throughout the surgery by a rectal probe and maintained at 37±0.5°C with a warming blanket. The left common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were successfully exposed and isolated through a midline cervical skin incision under a microscope. The ECA and CCA were ligated, and a loose slip-knot at the distal end of the ligation was made followed by the carotid artery was clamped and punctured with a 1 ml syringe needle at the bifurcation between the ligations. The nylon thread was then introduced and advanced into the ICA to a depth of approximately 18 mm to

occlude the MCA, the thread was fastened at the proximal end. Sham-operated rats were subjected to the same surgical procedure, while the nylon thread was not inserted to block blood flow into the MCA. After the surgery, the wound was sutured and rats were returned to their cage, the temperature was maintained at 29°C with free access to water and food. The neural scoring was evaluated on day 1, 14 and 28. Half of the rats were sacrificed on day 14 and the remaining subjects were killed on the day 28 after the scoring, the brain tissue were harvested and immediately frozen at -80°C until RT-PCR and Western blot analysis were completed, alternatively, the rats were transcardially perfused with 4% paraformaldehyde to evaluate the expression of Notch1 in situ.

Evaluation of the neurological scoring

The neurological scoring test was performed according to the classical Zea Longa methods [9] (five-pointscore) as follows: 0 points, no neurological deficit; 1 point, failure to fully extend the forepaw on the opposite side of the infarction, a mild focal neurological deficit; 2 points, crawling in a circular motion, a moderate focal neurological deficit; 3 points, falling to the opposite side of the infarction, a severe focal deficit; and 4 points, inability to walk, loss of consciousness and display of the 'Horner' syndrome, which is characterized by small rima oculi, miosis, and enophthalmos.

RNA preparation and quantitative RT-PCR

The mRNA expression levels of NICD, Hes1, Hes5 and Jag1 were determined by quantitative real-time PCR. Twenty-four rats (n = 8 per group) were sacrificed and the brain tissue was rapidly removed. Samples were stored at -80°C until the analysis of the mRNA or the protein was completed. Total RNA was extracted with Trizol reagent (Invitrogen, USA). cDNA was reverse transcribed with a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Quantitative real-time PCR was performed by applying the real-time SYBR Green PCR technology with the use of a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Validated primers were designed for each target mRNA. The primer pairs for NICD, Hes1, Hes5 and Jag1 and an internal standard (β-actin) were as follows: NICD forward, 5'-TCG GCA GCC TCA ATA TCC CCT ACA-3'; NICD reverse, 5'-TCTTGCTGGCC-TCTGACACTTTGA-3'; Hes1 forward, 5'-CGCCG-CCGCCGCTTGTGC-3'; Hes1 reverse, 5'-CCGA-GGTCCCGCTGTTGCTGGTGT-3'; Hes5 forward, 5'-CCAGGACTACAGCGAGGGTTACT-3'; Hes5 reverse, 5'-GGCAGATTGGCGCGAGGTGGAGAC-3' Jag1 forward, 5'-TGATGGGGCCAAGTGGGATGA-CGA-3'; Jag1 reverse, 5'-GGCTGGAGGCTGGA-GGACCGACAC-3; β-actin forward, 5'-CCTGTAC-GCCAACACAGTGC-3'; β-actin reverse, 5'-ATAC-TCCTGCTTGCTGATCC-3'.

Western blot analysis

An immunoblot analysis of the brain tissue was used to determine the expression levels of expression levels of NICD, Hes1, Hes5 and Jag1. The samples were lysed and homogenized in an RIPA lysis buffer (Beyotime, China) supplemented with PMSF (phenylmethanesulfonyl fluoride) on ice for 30 min. Then the total protein concentration was determined using a bicinchoninic acid kit (Beyotime, China). The resulting supernatants were separated with SDS-PAGE in 12% gel and transferred to PVDF membranes. The membrane was blocked with casein and probed with the appropriate dilution of antibodies against NICD (diluted 1:1000), Hes1 (diluted 1:1000), Hes5 (diluted 1:1000), Jag1 (diluted 1:1000) or β-actin (diluted 1:3000), followed by horseradish peroxidase-coupled detection.

Immunohistochemical analysis

The protein expression of α-SMA and Notch1 was determined by immunohistochemistry. Sixteen rats (n = 4 per group) were transcardially perfused with 4% paraformaldehyde, and the brain tissue were dehydrated, embedded in paraffin wax. A 5-mm section was deparaffinised in xylene and rehydrated through graded concentrations of ethanol. Theanti-Notch1 antibody was diluted to the concentration of 1:150, and finally, counterstaining was performed using haematoxylin. Negative control sections were prepared by replacing the primary.

The Immunohistochemical analysis was then performed with fluorescence microscope (Olympus, Japan) and the positive rate was analyzed by IPP software.

Statistical analysis

All of the above data were presented as mean ± SEM and statistical analysis was performed

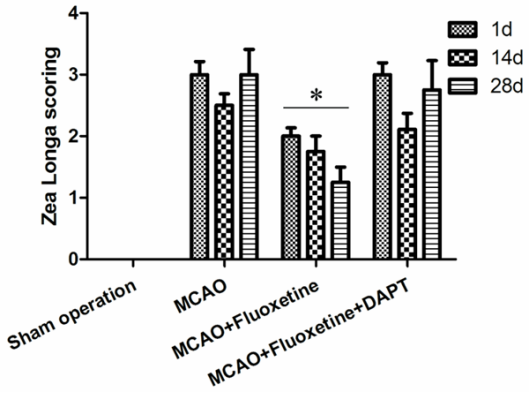

Figure 1. The neural scoring of different groups with Zea Longa scoring. MCAO treatment increased the score compared with the sham-operated group. Compared with the MCAO group, administration of fluoxetine attenuated the score significantly, while there is no difference between the MCAO group and the MCAO + DAPT. Data are expressed as the mean ± SEM of 12 rats from each group (*P < 0.05 vs. MCAO group).

with SPSS 16.0 software; The differences between groups were separately compared by analysis of variance (ANOVA) and P < 0.05 was considered the criterion for statistical significance.

Results

Fluoxetine alleviated MCAO-induce neurologic function damage

The neurologic functions were evaluated on days 1, 14, 28 after the surgery of MCAO shown in **Figure 1**. For the sham-operated group, the score is 0 on days 1, 14, 28. In the MCAO group, the Zea Longa score is 30 on days damage. icalh1a on days 1, 14, 28, respectively, which suggested that the occlusion of the middle cerebral artery induced severe neurologic function damage, in contrast, in the fluoxetine group, the Zea Longa score is 2±0.12, 1.75±0.23, 1.25±0.21 on days 1, 14, 28, and there is significant difference compared with the MCAO group (P < 0.05), which demonstrated that injection of fluoxetine attenuated the neurologic function damage. In addition, there are no remarkably differences between the MCAO group and fluoxetine + DAPT group G.

Fluoxetine and DAPT modulate the mRNA levels of NICD, Hes1, Hes5 and Jag1 in the brain tissue

To investigate the expression of key regulator of Notch1 signaling pathway after administration

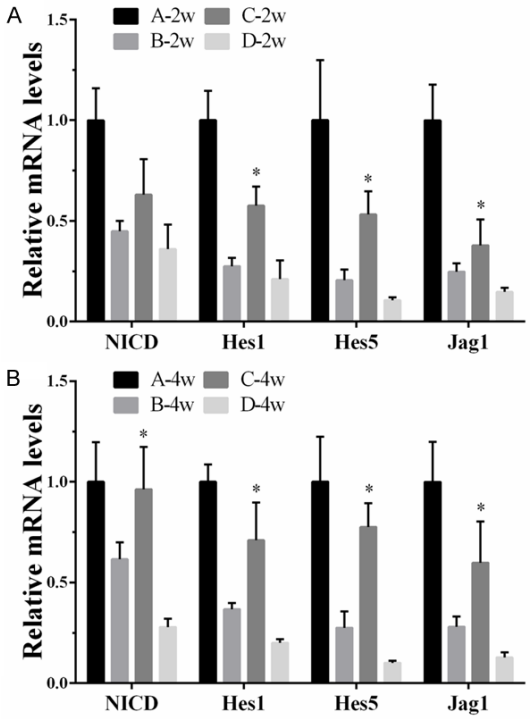

Figure 2. The mRNA levels of different groups with RT-PCR in 2 weeks (A) and 4 weeks (B). MCAO treatment decreased the mRNA levels of NICD, Hes1, Hes5 and Jag1 compared with the sham-operated group. Administration of fluoxetine increased the mRNA levels significantly compared with the MCAO group, while the DAPT treatment downregulated the mRNA levels. (six rats were sacrificed each time point per group, A represents Sham operation group, B represents MCAO group, C represents MCAO + Fluoxetine group, D represents MCAO + Fluoxetine + DAPT group, *P < 0.05 vs. MCAO group).

of fluoxetine and DAPT following the surgery of MCAO, as shown in **Figure 2**, quantitative real-time PCR experiments for NICD, Hes1, Hes5 and Jag1 were performed. The mRNA levels of NICD, Hes1, Hes5 and Jag1 were significantly increased when the rats were subjected to MCAO groups compared to the control group. In comparison with the MCAO group, NICD, Hes1, Hes5 and Jag1 expression in the fluoxetine groups was also increased. The mRNA levels of NICD, Hes1, Hes5 and Jag1 in the fluoxetine + DAPT group decreased significantly compared with the MCAO + fluoxetine groups.

Fluoxetine and DAPT modulate the protein levels of NICD, Hes1, Hes5 and Jag1 in the brain tissue

To investigate the expression of NICD, Hes1, Hes5 and Jag1 after the treatment of MCAO

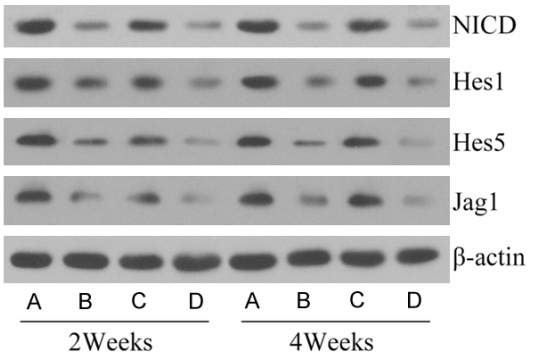

Figure 3. The protein levels of different groups with western blot. MCAO treatment decreased the protein levels of NICD, Hes1, Hes5 and Jag1 compared with the sham-operated group. Fluoxetine treatment increased the protein levels significantly compared with the MCAO group, while the DAPT treatment decreased the protein levels. (A represents Sham operation group, B represents MCAO group, C represents MCAO + Fluoxetine group, D represents MCAO + Fluoxetine + DAPT group, Six rats were sacrificed each time point per group, $^{*}P < 0.05$ vs. MCAO group).

and the drugs, western blot experiments for NICD, Hes1, Hes5 and Jag1 were performed as shown in **Figure 3**. The protein level of NICD, Hes1, Hes5 and Jag1 decreased significantly when the rats were subjected to MCAO groups compared with the sham-operated group. Compared with the MCAO group, NICD, Hes1, Hes5 and Jag1 expression in the fluoxetine groups rised remarkably, in addition, DAPT treatment decreased the level of NICD, Hes1, Hes5 and Jag1, there are remarkably differences between the fluoxetine groups and fluoxetine + DAPT group.

Fluoxetine and DAPT modulate the protein expression of Notch1 in the brain tissue

To further characterize the localization and protein expression of Notch1 in the brain tissue, immunohistochemistry analysis was performed. As shown in **Figure 4**. The MCAO operation caused notable declines in the protein expression of Notch1 compared to the sham-operated group, while Notch1 rised remarkably in the MCAO + fluoxetine group compared with MCAO group.

Discussion

In this study, we found that the administration of fluoxetine after the surgery of MCAO demonstrated a neuroprotective potential in rats, combined with modulation of Notch1 signaling pathway in the brain, however, the application of Notch1 signaling pathway inhibitor DAPT showed opposite outcomes.

As a classic antidepressant drug, fluoxetine also participated in recovery of cerebral ischemia disease. Chollet et al [10] reported that fluoxetine may be beneficial both in functional recovery and the prevention of post-stroke depression by modulating the spontaneous brain plasticity, Lim et al [11] also showed that fluoxetine improves motor impairment and neurological deficits by repression of microglia activation, neutrophil infiltration, proinflammatory marker expressions, as well as suppressed NF-kappa B activity. In our studies, we also reaffirmed that fluoxetine treatment alleviates motor impairment caused by MCAO operation in rats, however, our results also demonstrate that administration of fluoxetine was consistent with the modulation of Notch1 signaling pathway key proteins, which is different from the former mechanisms, what's more, when combined treat with fluoxetine, Notch1 signaling pathway inhibitor DAPT cannot lessen neural impairment resulted from MCAO, DAPT treatment also decreased the expression of NICD, Hes1, Hes5, Jag1, which not only confirms the protective role of fluoxetine in cerebral ischemic disease, but also strongly suggest that, at least in part, administration of fluoxetine can decrease the neurological scoring after the MCAO through the regulation of the Notch1 signaling pathway. The immunohistochemistry analysis of also showed that fluoxetine can decrease the expression of Notch1.

Givogri et al [12] has reported that Notch1 is expressed in neuroblasts and astrocytes within the subventricular zone. The Notch1 signaling pathway not only regulate neural stem cell behavior during development, but also play an important role in neurogenesis in the subventricular zone of normal and ischemic rat brain in vivo [13]. Oya et al [14] has shown that Notch signaling in the CA1 is activated in parallel with the increase of endogenous neural stem cells stimulated by ischemia, and that the attenuation of Notch signaling could induce more efficient differentiation of neural progenitors toward a neuronal lineage. The above studies have demonstrated that Notch1 signaling pathway are important factors involved post cranial

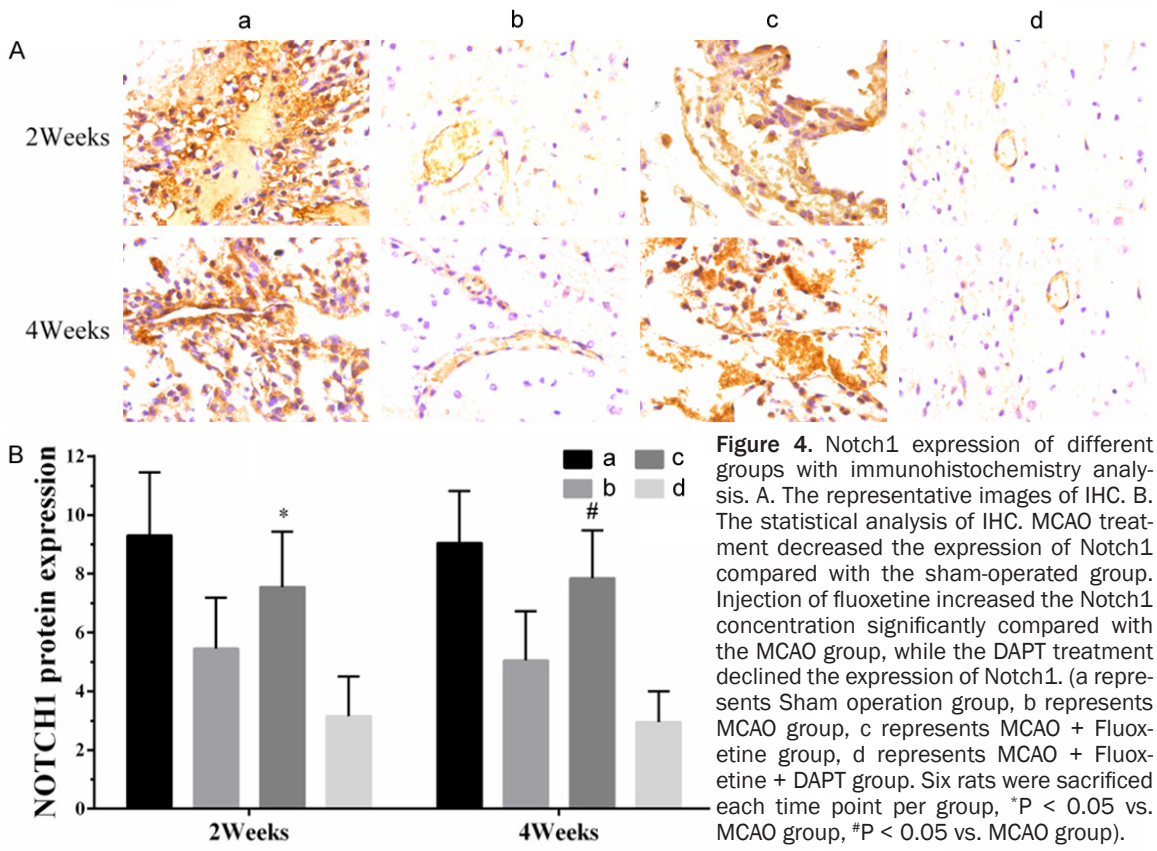

Figure 4. Notch1 expression of different groups with immunohistochemistry analysis. A. The representative images of IHC. B. The statistical analysis of IHC. MCAO treatment decreased the expression of Notch1 compared with the sham-operated group. Injection of fluoxetine increased the Notch1 concentration significantly compared with the MCAO group, while the DAPT treatment declined the expression of Notch1. (a represents Sham operation group, b represents MCAO group, c represents MCAO + Fluoxetine group, d represents MCAO + Fluoxetine + DAPT group. Six rats were sacrificed each time point per group, $^{*}P < 0.05$ vs. MCAO group, $^{\#}P < 0.05$ vs. MCAO group).

ischemic disease, after the establish of the MCAO models, our results demonstrated that after the stroke, Notch1 signaling pathway are regulated and suggested that fluoxetine treatment modulate the Notch1 signaling pathway, in addition, the Notch1 signaling pathway might be a potential target for the treatment of post-ischemic disease.

In summary, our study demonstrated that fluoxetine treatment alleviated cerebral ischemic disease in rats, probably by modulation of Notch1 signaling pathway, more detail mechanisms underlying the fluoxetine treatment should be probed and the key molecules should be screened and targeted.

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Disclosure of conflict of interest

None.

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