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XQ-1H alleviates cerebral ischemia in mice through inhibition of apoptosis and promotion of neurogenesis in a Wnt/β-catenin signaling dependent way

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ABSTRACT

Aims: 10-O-(*N*,*N*-dimethylaminoethyl)-ginkgolide B methanesulfonate (XQ-1H), a new derivative of ginkgolide B, has drawn great attention for its potent bioactivities against ischemia-induced injury. The purpose of this study was to further investigate the effect of XQ-1H against acute ischemic stroke by inducing middle cerebral artery occlusion/reperfusion (MCAO/R) injuries in mice.

Main methods: Treatment of XQ-1H (78 or 39 mg/kg, i.g., bid) 2 h after MCAO improved motor skills and ameliorated the severity of brain infarction and apoptosis seen in the mice by diminishing pathological changes and the activation of a pro-apoptotic protein Cleaved-Caspase-3, which in turn induced anti-apoptotic Bcl-xL. Through introducing Wnt/β-catenin signaling inhibitor XAV-939, XQ-1H was proven to intensively promoted neurogenesis in the peri-infarct cortex, subventricular area (SVZ) and the dentate gyrus (DG) subgranular area (SGZ) in a Wnt signal dependent way by compromising the activation of GSK3β, which in turn upregulated Wnt1, β-catenin, Neuro D1 and Cyclin D1, most possibly through the activation of PI3K/Akt signaling via the upregulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF).

Key findings: We conclude that XQ-1H preserved the motor functions, limited apoptosis, and concomitantly promoted neurogenesis-related protein expression by Wnt signaling-dependently compromising GSK3β/Caspase-3 activity and enhancing the expression of Wnt1/β-catenin/Neuro D1/Cyclin D1 and Bcl-xL.

Significance: This research may benefit the development of stroke therapeutics targeting neurogenesis through Wnt upregulation by XQ-1H.

1. Introduction

Ischemic stroke is a refractory disease due to low recovery rate [1]. Existing approved intravenous recombinant tissue plasminogen activators (rt-PA) [2] or intra-arterial mechanical thrombectomy [3] are limited by their narrow therapeutic window. Therefore, there is still an unmet need for new treatments.

In the acute phase of cerebral ischemia (minutes to hours after onset), blood-brain barrier (BBB) is impaired while the cells swell and dissolve, leading to edema and necrosis [4–6]. Necrotic debris causes many subacute events (hours to days), in which the ischemic nucleus extends to the penumbra, increasing the number of damaged and apoptotic neurons [7]. Many recovery processes occur during the chronic phase (weeks to months), in which neurogenesis has been a new focus in recent years [8,9].

Neurogenesis refers to the continuous production of new neurons from neural stem cells (NSCs) in specific areas (mainly the subventricular area (SVZ) and the dentate gyrus (DG) subgranular area (SGZ)) of the brain [10,11]. However, new neurons from adult neurogenesis are insufficient for tissue repair and functional recovery after stroke. Therefore, strengthening ischemic endogenous neurogenesis and promoting the survival of newborn neurons has presumably become a promising intervention for stroke treatment.

In adult brain stroke, neurogenesis is regulated by a variety of

Abbreviations: XQ-1H, 10-O-(*N*,*N*-dimethylaminoethyl)-ginkgolide B methanesulfonate; MCAO/R, middle cerebral artery occlusion/reperfusion; BBB, blood-brain barrier; SVZ, subventricular area; DG, dentate gyrus; SGZ, subgranular area; GSK3β, glycogen synthase kinase 3; PI3K, phosphatidylinositol-3-kinases; Akt, protein kinase B or PKB; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; rt-PA, recombinant tissue plasminogen activators; NSCs, neural stem cells; Axin, axis inhibition protein; BrdU, 5-bromo-2′-deoxypyridine; PFA, paraformaldehyde; TTC, 2,3,5-Triphenyltetrazolium chloride; EB, evans blue; H & E, Hematoxylin and eosin; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NeuN, neuronal nuclei; DCX, doublecortin; Bcl-xL, B-cell lymphoma-extra-large

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signaling pathways [12], in which canonical Wnt/β-catenin pathway is of great significance in cell survival after cerebral ischemia [13]. Nuclear import of β-catenin induced by Wnt ligands and PI3K/Akt mediated GSK-3β inactivation stimulates brain derived neurotrophic factor (BDNF) [14,15], nerve growth factor (NGF) [16] and several neurogenetic transcriptional factors including Cyclin D1 [17] and Neuro D1 [18], which facilitates brain protection, motor function recovery, neuronal survival and neurogenesis. TWS119 has been well known to have the capacity of inhibiting GSK-3β, which indirectly activates Wnt signaling ([19–21]; XAV-939, an inhibitor of tankyrase 1 and 2, stabilizes axis inhibition protein (Axin), thereby reinforcing the β-catenin destruction complex and serves as an indirect antagonist of Wnt signaling pathway [22] wildly used in Wnt pathway investigation [23,24]. Nevertheless, properties of TWS119 and XAV-939 in cerebral ischemia stroke have not been fully elucidated.

Ginkgolides are the main components in the preparation of *Ginkgo biloba* extract [25], in which Ginkgolides B (GB) has a therapeutic property on ischemic injury. Studies have shown that GB has a promoting effect on neurogenesis related to Wnt rescuing ([26,27]. Extensive evaluations have shown that 10-O-(*N*,*N*-dimethylaminoethyl)-ginkgolide B mesylate (XQ-1H, Supplementary Fig. 1), a new derivative of ginkgolide B, has a protective effect on cerebral ischemia injury in rats [28–30] and mice [31]. However, little has been mentioned regarding the effects and molecular mechanisms of XQ-1H on neuronal survival after stroke, especially neurogenesis in mice. On the other hand, the mice were treated with XQ-1H relatively frequently in previous studies, which was 3 times a day. Therefore, in this study, we investigated the neurofunctional prognosis, anti-apoptotic and neurogenetic effects of dose-adjusted XQ-1H in MCAO/R-injured mice, questioning whether Wnt/β-catenin related pathway activation was the potential mechanism of its action.

2. Results

2.1. XQ-1H limited cerebral infarction, brain edema, neurological deficits and BBB damage at 24 or 72 h after MCAO/R injury in mice

MCAO/R model was established in accordance with Fig. 1A. First, we investigated the protective effect of XQ-1H on cerebral ischemia injury in mice. XAV-939 was injected beforehand to explore whether the protective effect of XQ-1H on cerebral ischemia was connected with Wnt pathway. TWS119, as a GSK-3β inhibitor which has an indirect stimulating effect on Wnt pathway, was used as positive control. Similarly, Ginaton, a *Ginkgo biloba* extract was chosen to be a positive control because it's widely used for stroke treatment in China.

On the third day of administration, infarct area in MCAO/R model group (white) was obvious (Fig. 1B), accounting for about half area ($F(4, 49) = 24.292$, $p < 0.001$; 50.73%, Fig. 2E). The successful establishment of MCAO/R model was proved. The infarct volumes of XQ-1H at 78 mg/kg and 39 mg/kg (i.g., Bid.), Ginaton at 30 mg/kg (i.g., Bid.) and TWS119 at 30 mg/kg (i.p., Bid.) decreased significantly compared with model group ($p < 0.001$, Fig. 1B, D). The infarct area of the pathway inhibitor XAV-939 (10 mg/kg, i.p., Bid.) group and the XAV-939 + XQ-1H 78 mg/kg group were similar to that of the model group. At 78 mg/kg, XQ-1H had the lowest infarct volume rate (15.45%, Fig. 1C). Brain water content was measured as an indicator of edema ($F(5, 56) = 7.158$, $p = 0.003$). 78 mg/kg XQ-1H ($p < 0.001$), 39 mg/kg XQ-1H ($p = 0.029$) Ginaton and TWS119 ($p < 0.001$) significantly reversed the increased water content in the model group (Fig. 1E), and the inhibitory effect of XAV-939 pathway was consistent with the result of infarction rate. The effect of XQ-1H on BBB functional integrity after cerebral ischemia was characterized by Evans blue permeability test. Neurological scores and corner turn test were used to evaluate the effect of XQ-1H on the improvement of neural motor function after cerebral ischemia. XQ-1H showed similar effects with Ginaton and TWS119 in BBB integrity protection and neurological function improvement (Fig. 1F-H) and, and its effect can be inhibited by XAV-939. The results of infarct size and motor functions on the seventh day of administration were similar with the third day (Fig. 1C, D, F, G). It was tentatively concluded that XAV-939 could inhibit the Wnt pathway, and the effect of XQ-1H on cerebral ischemia may be relevant to this pathway.

2.2. XQ-1H improved pathological damage in hippocampus CA1, DG, cortex and SVZ regions at 72 h after MCAO/R injury in mice

After a three-day administration, H&E staining was used to assess the pathological damage of brain tissue of different regions after MCAO/R in mice. As shown in Fig. 2A, hippocampus CA1, DG, Cortex and SVZ region were located and evaluated respectively. No remarkable neuronal abnormalities were observed in the sham group. The MCAO/R group showed evident pathological damage, such as loose cortical interstitial, foamy softening structure and neuronal degeneration including nuclear pyknosis, shrinkage and neuronal loss in cerebral cortex and hippocampus. XQ-1H 78 mg/kg administration recovered the morphology and the structure of cells (Fig. 2A), as well as improving pathological scores in CA1 (0.67 ± 0.577, $F(6, 56) = 15.437$; $p = 0.004$) and cortex (1.44 ± 0.882, $F(7, 55) = 1.872$; $p = 0.008$) regions (Fig. 2B-E). Simultaneously, XQ-1H 39 mg/kg, TWS119 and Ginaton attenuated MCAO/R-induced pathological change seen in model rats and reduced the pathological scores in four regions especially CA1 and cortex areas (Fig. 2B-E), while XAV-939 and XAV939 + XQ-1H groups showed no difference with model group.

2.3. XQ-1H reduced the apoptosis rate of neurons and regulated the expression of related proteins at 72 h after MCAO/R injury in mice

TUNEL staining of the ischemic side of the brain after MCAO/R showed a significant enhancement in apoptosis, which was weakened by XQ-1H treatment in two dose groups ($F(5, 130) = 59.651$; $p < 0.001$, Fig. 3A, B).

Similarly, XQ-1H 78 mg/kg ($p = 0.036$) and Ginaton ($p = 0.007$) had a significant up-regulation effect on brain anti-apoptotic molecule, B-cell lymphoma-extra-large (Bcl-xL, $F(5, 24 = 4.380)$, $p = 0.003$) [32] after MCAO/R (Fig. 3C, D), which was opposite to Cleaved caspase-3 ($F(5, 24) = 5.778$, $p = 0.001$; XQ-1H 78 mg/kg, $p = 0.008$; Ginaton, $p = 0.035$; Fig. 3C, E). XAV-939 disrupted the effect of XQ-1H. These results further indicated the vital role of Wnt/β-catenin pathway in cell survival after MCAO/R and the protection activity of XQ-1H against apoptosis.

2.4. XQ-1H activated Wnt/β-catenin pathway at 72 h after MCAO/R injury in mice

It has been well known that Wnt/β-catenin signaling pathway plays an important role in cell growth, proliferation, especially in neuronal survival [33] with Wnt1, β-catenin, GSK-3β its important parts. Wnt1, as extracellular ligand, activates Wnt pathway through inactivation of GSK-3β contained polymer, leading to GSK-3β phosphorylation (Ser-9 coli of GSK–3β is one of its vital beneficial phosphorylation sites) and loss of inhibitory effect on β-catenin. β-catenin can then enter the nucleus and play its role in promoting transcription of key downstream genes. In order to thoroughly investigate the relationship between effect of XQ-1H on cerebral ischemic injury and Wnt/β-catenin pathway, we applied immunofluorescence, PCR and Western blotting techniques to evaluate the expression of key component in Wnt pathway.

The expression of β-catenin in SVZ, a key region of neurogenesis by immunofluorescence. In Fig. 4A, β-catenin expression in the two dose groups of XQ-1H, TWS119 and Ginaton groups was more obvious than that in the MCAO/R group, which could be reversed in XAV-939 and XQ-1H + XAV-939 group. Consistently, 78 mg/kg XQ-1H, Ginaton and TWS119 significantly enhanced the mRNA expression of Wnt1 ($F(5, 73) = 352.385$; $p < 0.001$) and β-catenin ($F(7, 16) = 74.043$;

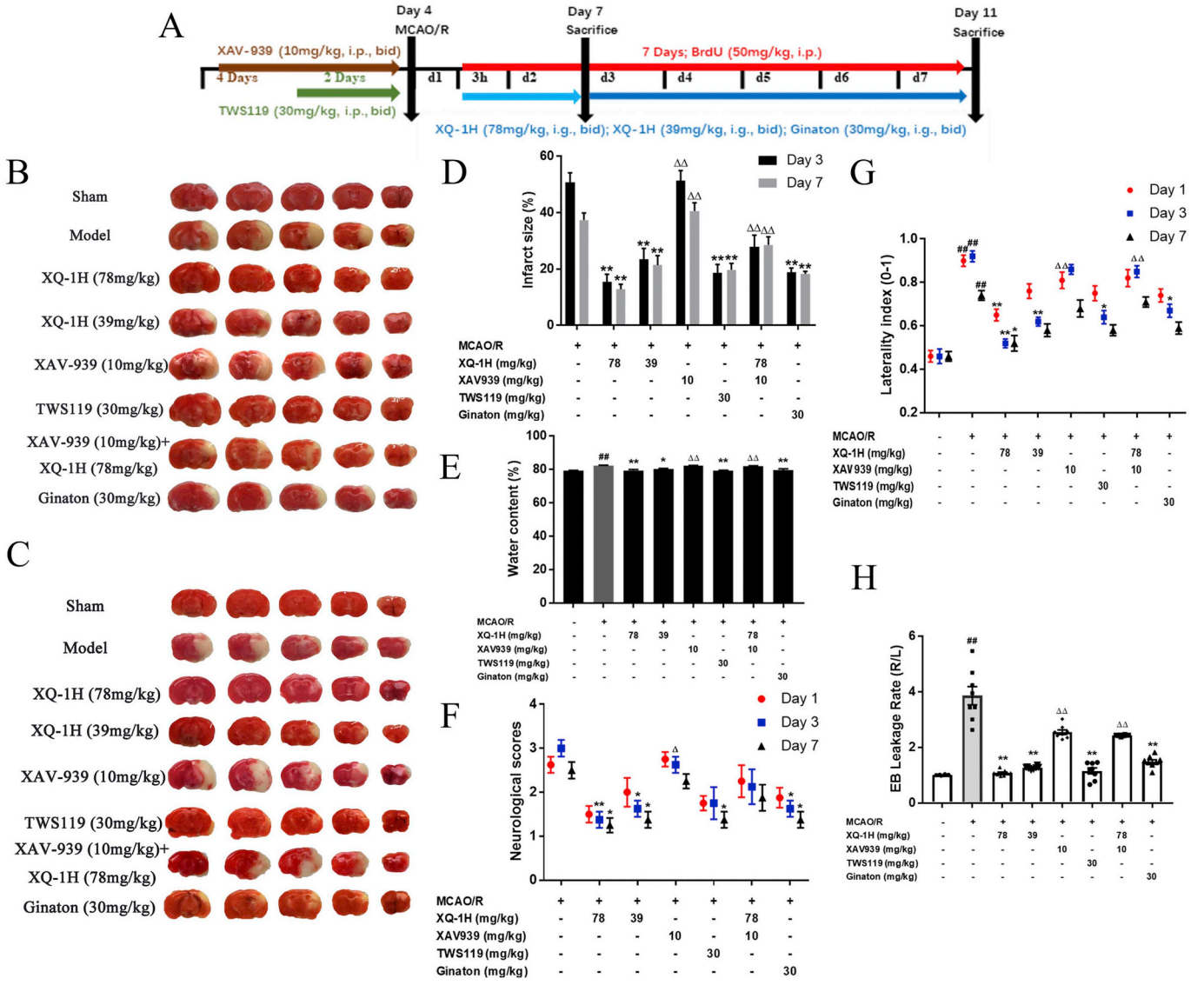

Fig. 1. Effects of XQ-1H on changes in brain infarction, water content, BBB dysfunction and neurological deficits after ischemic stroke injury. (A) Schematic diagram of BrdU and drug administration before/after MCAO/R; (B, C) Representative images of cerebral infarct area after cerebral ischemia at the third and seventh day; (D) Quantification of cerebral infarct area; (E) Quantification of cerebral edema at the third day; (F, G) Behavioral scoring and corner turn test statistics at the first, third and seventh day; (H) Quantification of BBB integrity in MCAO/R mice at the third day. With $n = 8$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with Kruskal-Wallis test for the neurological scores and corner turn test and two-way ANOVA followed by Bonferroni test for the remaining data. Animals were divided into various sub-groups, including mice from among the sham-operated mice (Sham); mice post-treated with vehicle (MCAO/R), with XQ-1H (78 or 32 mg/kg, i.g., bid; XQ-1H 78 mg/kg or 39 mg/kg) or Ginaton (30 mg/kg, i.g., tid; Ginaton 30 mg/kg), pre-treated with XAV-939 (10 mg/kg, i.g., bid; XAV-939 10 mg/kg), TWS119 (30 mg/kg, i.g., bid; TWS119 30 mg/kg) or pre-XAV-939 combined with post-XQ-1H (XAV-939 10 mg/kg + XQ-1H 78 mg/kg) undergoing ischemia stroke.

$p < 0.001$), and inhibited the mRNA level of GSK–3β mRNA (F (5, 20) = 41.916; $p < 0.001$, Fig. 2B). As a Wnt pathway inhibitor, XAV-939 as well as XAV-939 + XQ-1H group had no significant difference with MCAO/R group ($p > 0.05$, Fig. 2C).

Similarly, compared with MCAO/R group, the cytoplasmic (F (5, 24) = 7.634; $p = 0.001$) and nuclear entry of β-catenin (F (5, 24) = 16.515; $p < 0.001$, Fig. 4C, D, G), p-GSK–3β/GSK–3β ratio (F (5, 24) = 3.621; $p = 0.013$, Fig. 4C, E), and Wnt1 protein content (F (5, 24) = 3.917; $p = 0.018$, Fig. 4C, F) were significantly higher in XQ-1H 78 mg/kg group ($p < 0.05$), and the effects were restrained by XAV-939. These results, together with those in Figs. 1 and 2, implicated that XAV-939 inhibited the Wnt/β-catenin pathway and that dosage-adjusted XQ-1H at 78 mg/kg could be used to treat cerebral ischemia in mice by activating this pathway.

2.5. XQ-1H boosted neurogenesis in SVZ, DG and peri-infarct areas at 7 days after MCAO/R injury in mice

To investigate the effect of XQ-1H on neurogenesis in mice, we used BrdU (green dots) and NeuN (red dots) to co-locate newly proliferated neurons in SVZ, DG and cortical regions 7 days after MCAO/R injury and BrdU injection (F (7, 16) = 34.453, $p < 0.001$; Fig. 5A, B). The number of $BrdU^+$ neurons in SVZ area of MCAO/R group slightly increased compared with that in the sham group, but there was no significant difference ($p = 1.000$). $BrdU^+$ neurons highly presented in groups of 78 mg/kg XQ-1H ($p < 0.001$; $p = 0.002$), TWS119 ($p = 0.003$) and Ginaton ($p < 0.001$), with no differences in XAV-939 group ($p = 1.000$) and combined group ($p = 1.000$). In the same way, $BrdU^+$ Neurons appeared more apparently in hippocampus DG area especially in 78 mg/kg XQ-1H group (F (5, 52) = 31.854, $p < 0.001$,

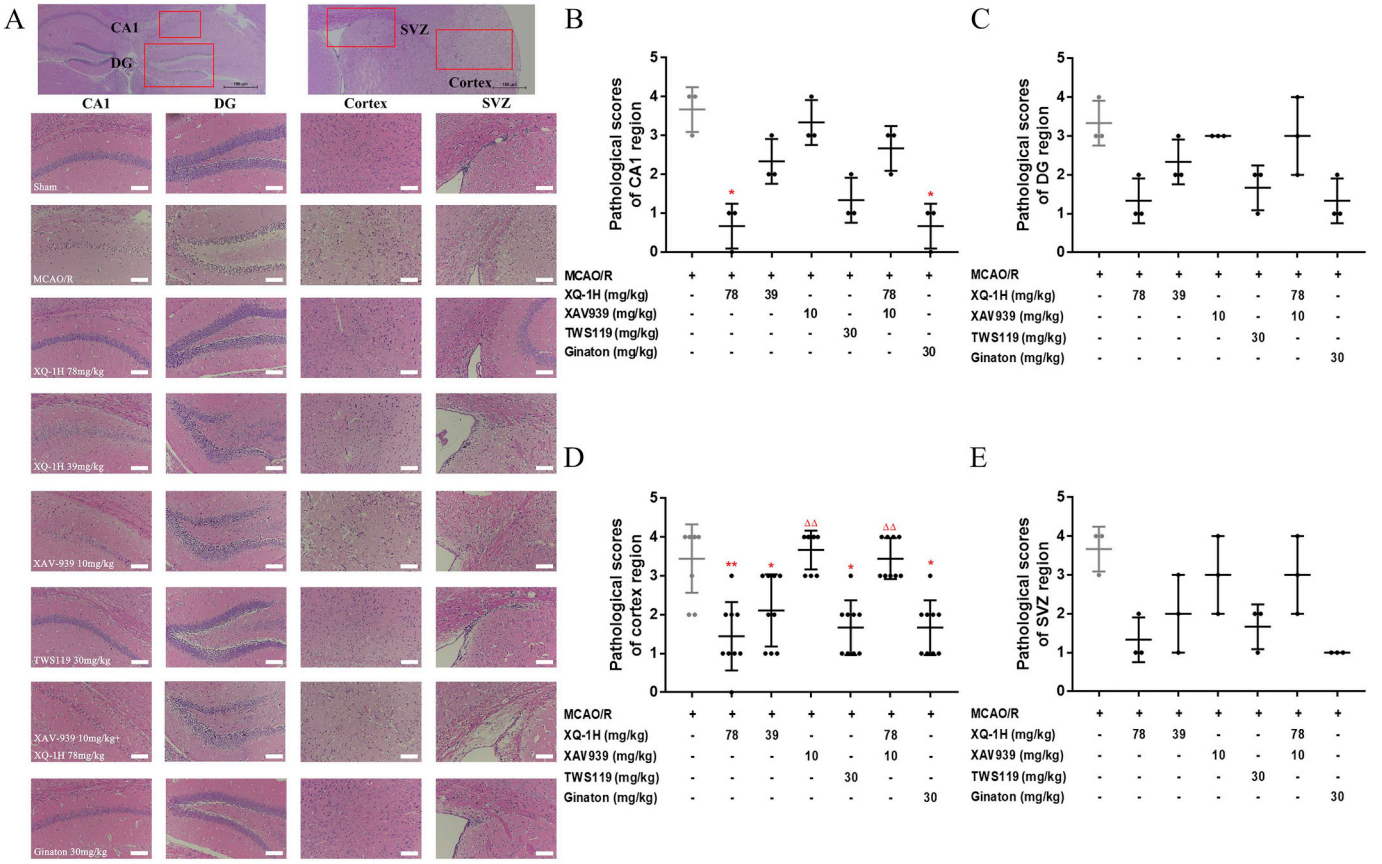

Fig. 2. Th Effects of XQ-1H on pathological changes in hippocampus CA1, dentate gyrus (DG), cortex and subventricular (SVZ) areas in mice (at 72 h) after ischemic stroke injury. (A) Representative images of pathological changes in different brain regions; (B-E) Pathological scores of different brain regions. With $n = 3$ mice per group and $n = 9$ for cortex region since three areas of each mouse was selected. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with Kruskal-Wallis test. Scale bar is 100 μm in the images.

Fig. 5C, D). Consistent conclusions could be obtained in cortex area (Fig. 5E, F). These results revealed that XQ-1H promoted post-stroke neurogenesis and activated Wnt/β-catenin signaling pathway, which restored neurogenesis deficit in the XAV-939 administration.

Meanwhile, we did some investigation on functional recovery at this time point. XQ-1H showed significant effects in infarction rate and neurological function improvement (Fig. 6, A-C), and its effect can be inhibited by XAV-939, identical to the results at day 3.

2.6. XQ-1H promoted endogenous neurogenesis through modulation of neurogenetic signaling at 72 h after MCAO/R injury in mice

To further explore whether endogenous neurogenesis could be promoted by XQ-1H, TWS119 and/or Ginaton treatments at 72 h after stroke, we used doublecortin (DCX, a marker for newborn neuroblasts [34,35]) as an indicator to elucidate early phase endogenous neurogenesis and detected the expression of neurogenesis-related proteins. ELISA examination showed obvious increase in BDNF and NGF, neurotrophic factors which facilitate neurogenesis, in 78 mg/kg XQ-1H as well as TWS119 and Ginaton groups (F (5, 56) = 16.022; F (5, 56) = 24.335; $p < 0.001$, Fig. 6A, B) compared with MCAO/R group (BDNF: 182.32 ± 24.45 pg/mL; NGF: 39.44 ± 5.15 pg/mL), among which 78 mg/kg XQ-1H increased BDNF and NGF to 300.72 ± 53.11 pg/mL and 78.26 ± 14.09 pg/mL respectively. The mice in MCAO/R group showed relatively higher staining levels of DCX within DG region as compared to the sham-operated group (F (7, 60) = 24.263; $p = 0.043$). Treatment with 78 mg/kg XQ-1H ($p < 0.001$), TWS119 ($p < 0.001$) and Ginaton ($p = 0.015$) upregulated DCX expression as compared to the MCAO/R group (Fig. 6C, D), while XAV-939 and XAV-939 + XQ-1H groups had no difference with MCAO/R group ($p = 1.000$). Our results of Western blotting showed that treatment with 78 mg/kg XQ-1H significantly rescued the expression level of p-Akt (an upstream signal for p-GSK3β, F (5, 16) = 6.149; $p = 0.001$) and its upstream signal brain-derived neurotrophic factor (BDNF, F (5, 24) = 14.502; $p < 0.001$) [36] in a manner that paralleled the activation of Wnt1, p-GSK3βand cytoplasm/nuclear β-catenin (Fig. 4), accompanied by augment in the levels of Neuro D1 (F (5, 16) = 5.703; $p = 0.02$) and Cyclin D1 (F (5, 24) = 7.222; $p = 0.001$), two downstream transcriptional factors of Wnt signaling (Fig. 6E-J), which were also repressed by XAV-939. These results further proved the conclusion in 2.5 that XQ-1H promoted both early stage and sub-acute stage neurogenesis after ischemic injury in a Wnt/β-catenin pathway dependent manner.

3. Discussion

In this study, we induced ischemic injury in mice by MCAO/reperfusion surgery to prove for the first time that XQ-1H alleviated cerebral ischemic injury through anti-apoptotic and neurogenetic activities. And its effects were dependent on activating Wnt/β-catenin signaling pathway.

Currently, *Ginkgo biloba* standardized extract (EGb 761, or Ginaton in China) has been widely used in the treatment of stroke in China [37]. We used Ginaton as one of the positive controls because latest studies found that EGb 761 could promote cerebral neurogenesis in mice [26,27]. In the study, we revealed that XQ-1H at a dose of 78 mg/kg (twice a day, at an interval of 12 h) had the same effect as that of Ginaton on the prognosis of cerebral ischemia in mice, exhibited by

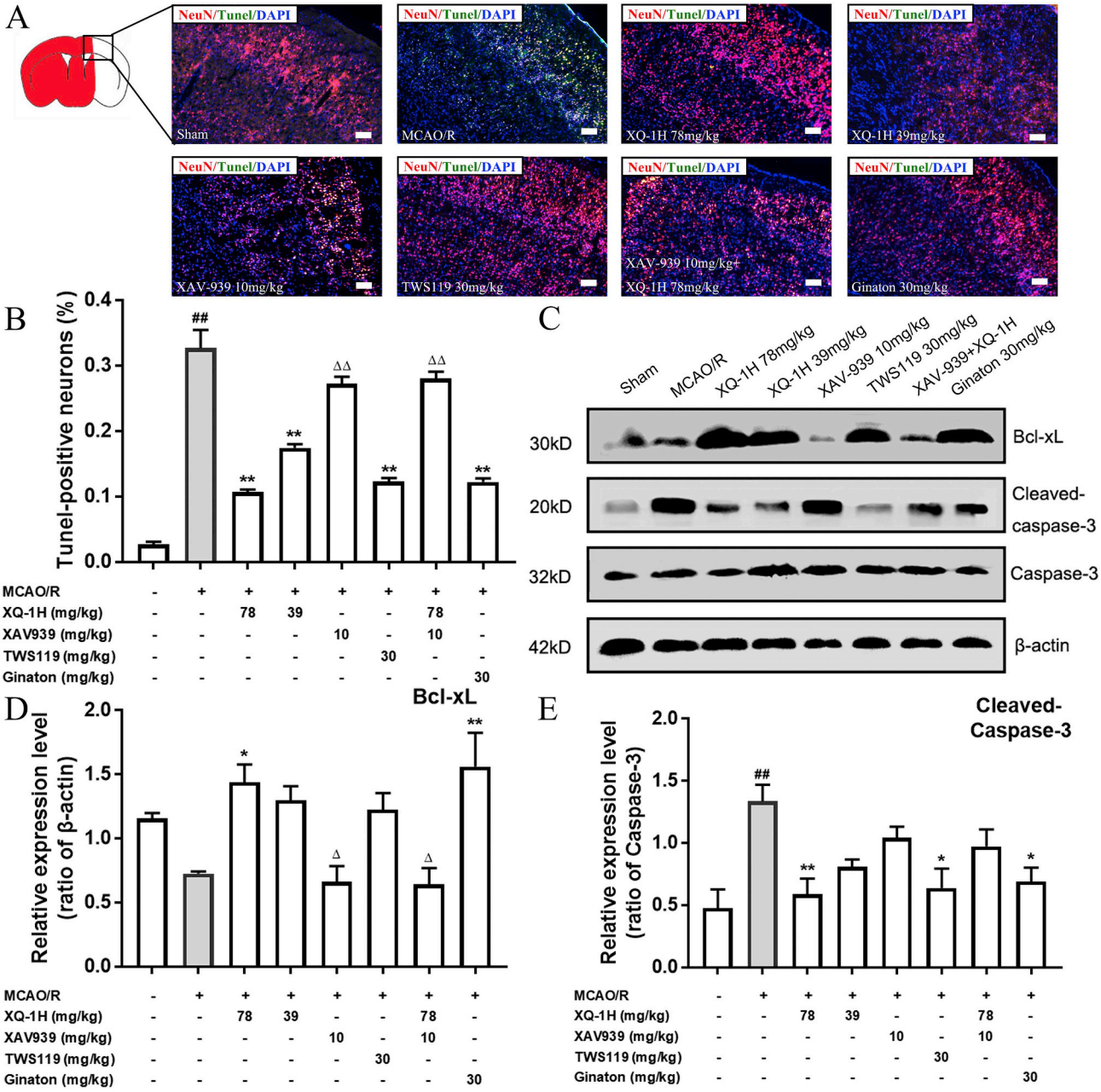

Fig. 3. The effect of XQ-1H on the apoptosis rate and related protein expression in mice (at 72 h) after ischemic stroke injury. (A, B). Apoptosis rate images and quantification of neurons in infarcted cortical area; (C-E) Typical Western blotting analysis using brain tissue from the ipsilateral ischemic brain hemisphere was conducted to show changes of apoptosis-related proteins Bcl-xL, Cleaved Caspase-3/total Caspase-3 in cerebral area; β-actin was included as a reference for normalization; with $n = 3$–5 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with two-way ANOVA followed by Bonferroni test. Scale bar is 100 μm in the images.

decreased cerebral infarction rate and water content, blood-brain barrier protection, and ameliorated neuromotor functions and pathological injury (Figs. 1, 2). This protective effect of XQ-1H was achieved by reducing the degree of apoptotic cell death through a regulation in apoptotic associated cascade of Cleaved-Caspase-3 and Bcl-xL (Fig. 3). Pro-apoptotic protein Cleaved caspase-3 indicates early apoptosis [38], while Bcl-xL belongs to the Bcl-2 family which inhibits apoptosis [32]; XQ-1H also promoted endogenous neurogenesis through the upregulation of β-catenin and its downstream protein Neuro D1 and Cyclin D1 by suppressing GSK3β activity and enhancing upstream PI3K/Akt signaling through activation of BDNF and NGF pathway (Fig. 4-6). Moreover, the results of the present study showed that treatment with Wnt pathway agonist TWS119 provided less of a protective effect than treatment with XQ-1H; this could be due to the lack of any activity caused by TWS119 in terms of PI3K/Akt activation and GSK3β inactivation (Fig. 7).

The Wnt/β-catenin signaling pathway plays an important role in many cellular processes, including survival, proliferation and cell fate [39]. In order to further explore whether the neuroprotective mechanism of XQ-1H was related to the Wnt pathway, we selected XAV-939 as the inhibitor of the Wnt pathway. After administration of XQ-1H and TWS119 (30 mg/kg, i.p., bid.), the expression of β-catenin (paramount component of Wnt pathway) increased in the key area of neurogenesis SVZ (Fig. 4A, B). The regulation effects of XQ-1H on Wnt1, β-catenin and GSK-3β (Fig. 4B) in RT-PCR and Western Blot (Fig. 4C-G) were reversed by XAV-939 (10 mg/kg, i.p., bid.). Two-way ANOVA

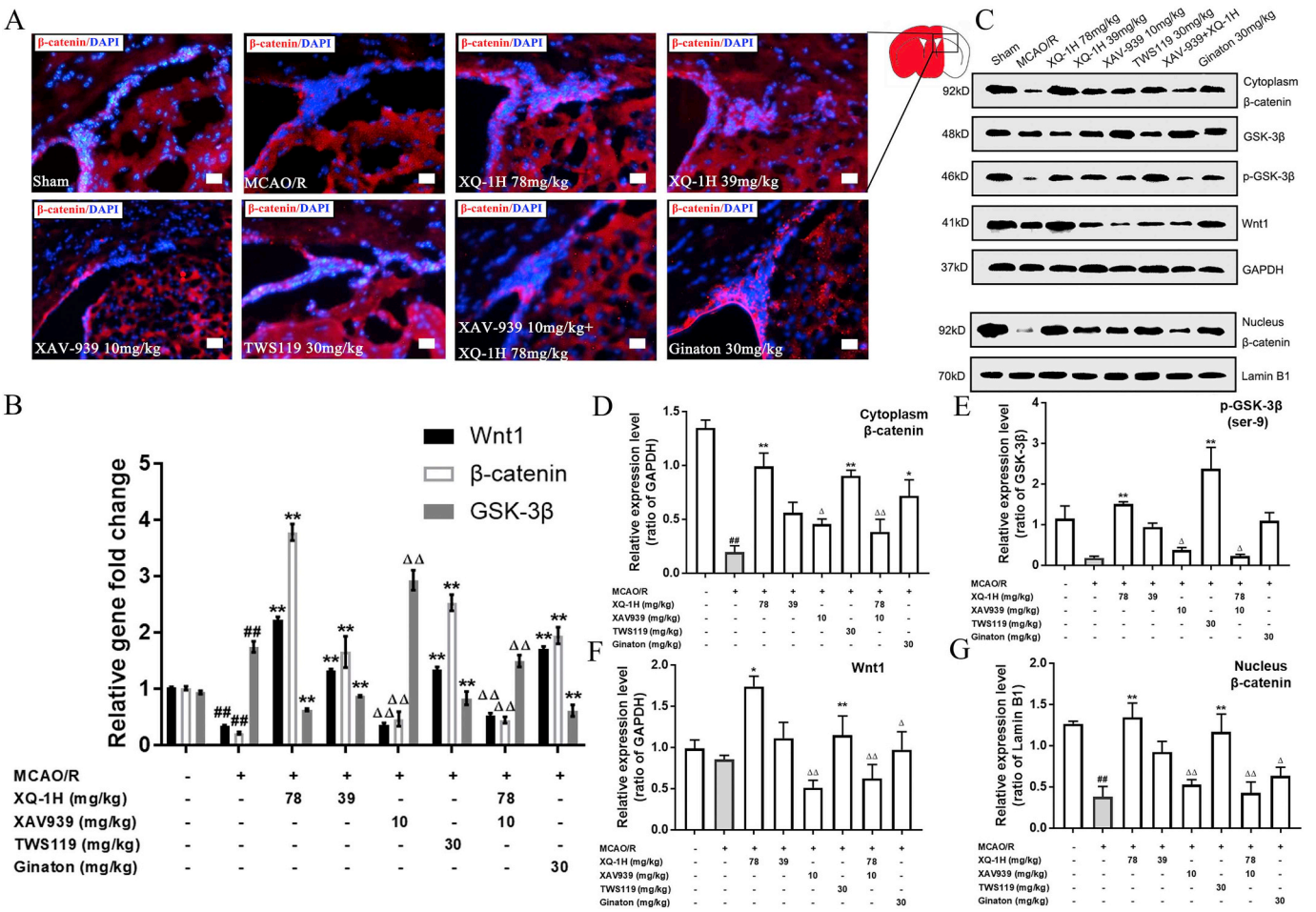

Fig. 4. The effect of XQ-1H on the expression of Wnt/β-catenin pathway components in mice (at 72 h) after ischemic stroke injury. (A) Representative images of Expression of β-catenin in SVZ region; (B) mRNA expression and quantification of key components in Wnt/β-catenin signaling pathway: β-catenin, Wnt1, GSK-3β; (C-G) Wnt1, p-GSK-3β/GSK-3β protein levels, and nucleation of β-catenin protein and quantification; GAPDH or Lamin B1 was included as a reference for normalization; With $n = 3$–5 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with Mann-Whitney *U* test for real-time PCR and two-way ANOVA followed by Bonferroni test for other data. Scale bar is 10 μm in the images.

showed no significant interaction between XQ-1H and XAV-939 on the expression of Wnt/β-catenin components in each group after cerebral ischemia injury. Therefore, the role of XQ-1H or XAV-939 was considered to be independent. As a result, the therapeutic effects of XQ-1H after cerebral ischemia/reperfusion were associated with activation of the Wnt/β-catenin signaling pathway.

Recent experimental findings suggest that enhancing endogenous neurogenesis in adult SVZ and SGZ may be an additional mechanism contributing to the improved neurological outcomes after stroke [40], in which Wnt signaling plays a critical role [35]. Our results showed that the number of $BrdU^{+}$ and DCX^{+} neurons in the MCAO/R group was enhanced not sufficiently in SVZ, SGZ or cortex areas of the brain. XQ-1H 78 mg/kg treatment increased the ratio of $BrdU^{+}$ (7 days of treatment) and DCX^{+} (3 days of treatment) neurons (Fig. 5, 6C, D), which revealed that XQ-1H induced both sub-acute and chronic neurogenesis in SVZ and SGZ.

One possible mechanism of cerebral ischemic neurogenesis is the stimulation of tyrosine kinase-coupled receptors by growth factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) [41,42]. Studies have shown that BDNF and NGF correlate with Wnt pathway to promote cell survival and neurogenesis [14–16]. Neuro D1 and Cyclin D1 are both downstream transcriptional factors of Wnt/β-catenin and are favorable to neurogenesis. Neuro D1 is a transcription factor that promotes the development of NSCs neurons [18,43], and Cyclin D1 is a cell cycle checkpoint protein, which plays a role in regulating the proliferation of cells ([17,44]. Our results showed that XQ-1H promoted neurogenesis and cell proliferation by up-regulating the expressions of BDNF, NGF, Neuro D1 and CyclinD1, together with activation of Wnt pathway via GSK-3β inhibition through PI3K/Akt activation (Fig. 6). XAV-939 abolished the above cell anti-apoptosis and regeneration promotion effects. These results certified that in cerebral ischemia injury, XQ-1H exerted anti-apoptotic and neurogenic properties, which may be activated by promoting the Wnt/β-catenin signal pathway.

4. Conclusion

In summary, we evaluated whether XQ-1H exerted neuroprotective, neurogenesis promotion and anti-apoptotic effects cerebral ischemia reperfusion injury in mice via the Wnt/β-catenin signaling pathway. The results showed that GSK-3β and β-catenin activation by XQ-1H was induced through BDNF and PI3K/Akt pathway upregulation, which brought about anti-apoptotic and neurogenetic reactions via regulation of relevant components. And introduction of Wnt signaling inhibitor XAV-939 further revealed that XQ-1H ameliorated ischemic stroke in a Wnt/β-catenin signaling dependent way. Therefore, XQ-1H might be selected as a potential therapeutic drug for neuronal protection after stroke, providing a theoretical basis for future clinical applications.

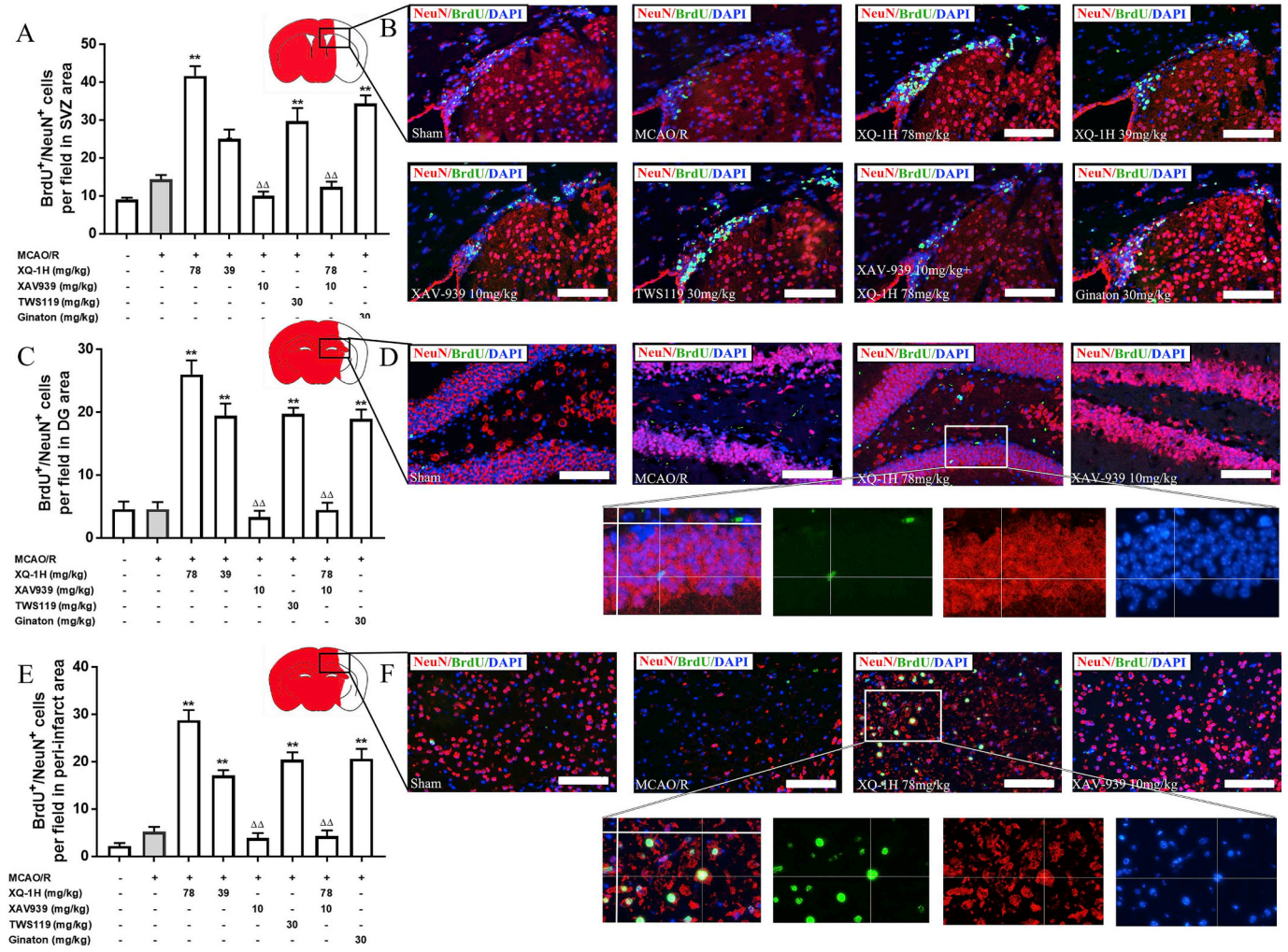

Fig. 5. The effect of XQ-1H on endogenous neurogenesis in hippocampus CA1, DG and cortex regions in mice (at 7th day) after ischemic stroke injury. (A, B) Representative images of BrdU$^+$ neurons in SVZ region and its quantization; (C-D) Representative images of BrdU$^+$ neurons in DG region and its quantization; (*E*-F) Representative images of BrdU$^+$ neurons in cortex region and its quantificaiton; with $n = 3$–5 mice per group. $^*p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with two-way ANOVA followed by Bonferroni test. Scale bar is 100 μm in the images.

5. Methods and materials

5.1. Animals and treatments

450 adult male C57BL/6 mice (22–25 g, Beijing Vital River Laboratory Animal Technology Co., Ltd.) were maintained 5 per cage in plastic bottomed cages containing wood shavings as bedding in a temperature-controlled room (22 ± 2 °C), with a 12 h light-dark cycle and a relative humidity of 60 ± 10%. Mice were allowed free access to food and water and were fasted for 12 h before surgery. After surgery, the mice were randomly divided into 8 groups: Sham, MCAO/R, XQ-1H (39 mg/kg), XQ-1H (78 mg/kg), XAV-939 (10 mg/kg), TWS119 (30 mg/kg), XAV-939 (10 mg/kg) + XQ-1H (78 mg/kg) and Ginaton (30 mg/kg). For evaluation of infarct size, brain edema, BBB integrity, neurological deficiency and ELISA analysis, $n = 8$ for each group. For immunofluorescence, H & E staining, real time PCR and Western Blotting analysis, $n = 3$ for each group. All animals were cared for in compliance with institutional guidelines of China Pharmaceutical University (Nanjing, China). All efforts were made to minimize animal suffering and reduce the number of animals used. Four mice died from anesthesia and fifteen died from intracranial hemorrhage, and the data from these mice were excluded in the analysis.

5.2. Drug administration

XQ-1H (99.35% by HPLC, Jiangsu Kefeiping pharmaceutical co., LTD.) was dissolved in normal saline (0.9% NaCl). Mice were given different doses of XQ-1H (78 mg/kg, 39 mg/kg), with volume of 0.1 mL/10 g, twice a day starting from 2 h after MCAO. The administration interval was 12 h, with some modulation of previous studies [45]. XAV-939 (β-catenin inhibitor, Medchem expression, Shanghai, China; Cat# HY-15147 [23,24]) and TWS119 (GSK-3β specific inhibitor, Wnt/β-catenin pathway agonist, Medchem said, Shanghai, China; Cat# HY10590 [19–21]) were dissolved in DMSO and then diluted with normal saline. XAV-939 were injected intraperitoneally with 30 mg/kg at 0.1 mL/10 g for 4 days before surgery, twice a day, and the last injection was at 1 h before surgery. TWS119 were injected with 10 mg/kg intraperitoneally 2 days before operation at MCAO (0.1 mL/10 g), twice a day, and the last injection was at 1 h before operation (Fig. 1). Use 0.5% carboxymethyl cellulose sodium (CMC−Na) to dissolve Ginaton ((Dr. Willmar Schwabe GmbH) with the dose of 30 mg/kg and administrate 3 times a day each 8 h.

5.3. Focal cerebral ischemia establishment

Transient MCAO/R injury was used to mimic ischemic stroke injury as previously described [46,47]. Mice were anesthetized with 2%

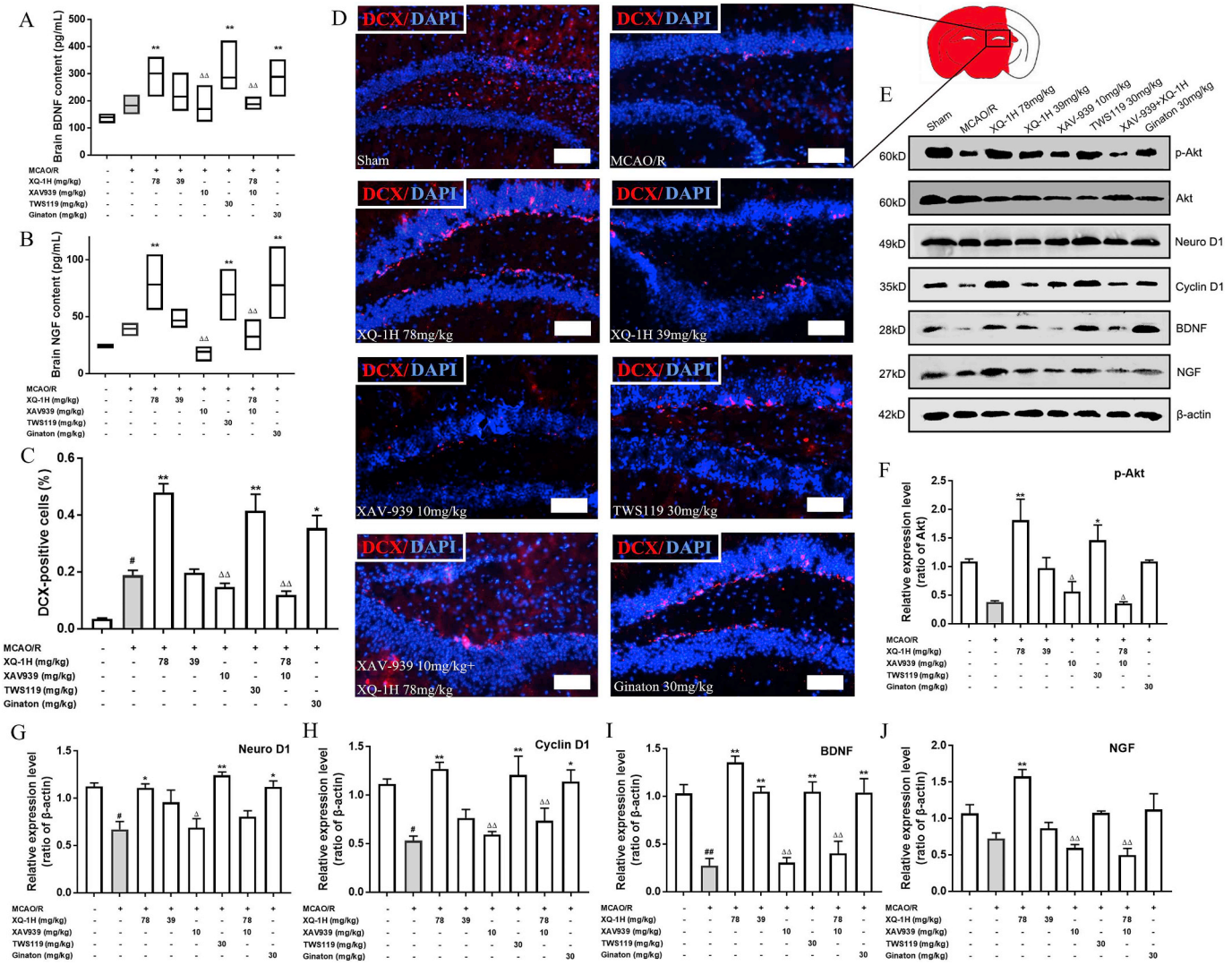

Fig. 6. Effect of XQ-1H on expression of neurogenesis relevant components in mice (at 72 h) after ischemic stroke injury (A, B) Concentration of BDNF and NGF in mice brain by ELISA tests; (C, D) Quantification and representative images of XQ-1H on the expression of DCX^+ newborn neuroblasts; (E-J) Protein expression images and quantification of neurogenesis related proteins p-Akt/Akt, Neuro D1, Cyclin D1, BDNF and NGF; with $n = 3–5$ mice per group. $^*p < 0.05$, $^{**}p < 0.01$ vs. MCAO/R, $\#p < 0.05$, $\#\#p < 0.01$ vs. Sham; $^{\triangle}p < 0.5$, $^{\triangle\triangle}p < 0.01$ vs. XQ-1H 78 mg/kg with two-way ANOVA followed by Bonferroni test. Scale bar is 100 μm in the images.

chloral hydrate (0.2 mL/10 g, i.p.) and fixed in supine position. In short, we inserted a silicon-coated monofilament nylon suture (Yushun Biotechnology co., LTD. Pingdingshan, China; Cat#0624) about 0.16 mm in diameter to block the origin of MCA. The indwelling suture was placed for 45 min before removed for reperfusion. Throughout the experiment, mice body temperature was maintained at 37 ± 0.5 °C. The sham group underwent the same procedure without occlusion of the MCA. All the other groups underwent MCAO/R surgery.

5.4. In vivo labeling of 5-bromo-2′-deoxypyridine (BrdU)

BrdU (50 mg/kg in normal saline, daily, i.p., Sigma-Aldrich, St. Louis, MO, USA; Cat# B5002; Lot# HMBF1678) was given 7 days after MCAO (Fig. 1). Animals were deeply anesthetized (2% chloral hydrate) and transcardially perfused with normal saline (50 mL) and then 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 (50 mL). Tissue was post-fixed in PFA for 48 h and then placed in a gradient of sucrose (20 and 30%) for dehydration. Brains were flash frozen and then coronally sectioned (15 μm thick) in a stereological series, embedded in O.C.T. compound and stored at −80 °C.

5.5. Assessment of neurological defects

Neurological defects were measured according to the method of Bederson [48]. 1 h after the second, third and seventh day of administration. The mice were hung by the tail above the floor to observe the flexion of the forelimbs. 0 = normal activity, no neurological impairment; 1 = the left front claw is bent and adducted, and the tail is lifted and attached to the chest; 2 = reduction of lateral pushing resistance; 3 = unilateral rotation when walking freely; 4 = no spontaneous walking and flaccid paralysis. The higher the score, the worse the neurological function.

5.6. Corner turn test

In corner turn test [49,50], mice were placed in an equipment made by two vertical board with an angle of 30°, and the laterality index (defined as number of right turns/ten measurements) was evaluated. A score of 1 indicated severe motor deviation due to neurological impairment caused by stroke, and a score of 0.5 indicated no neurological impairment.

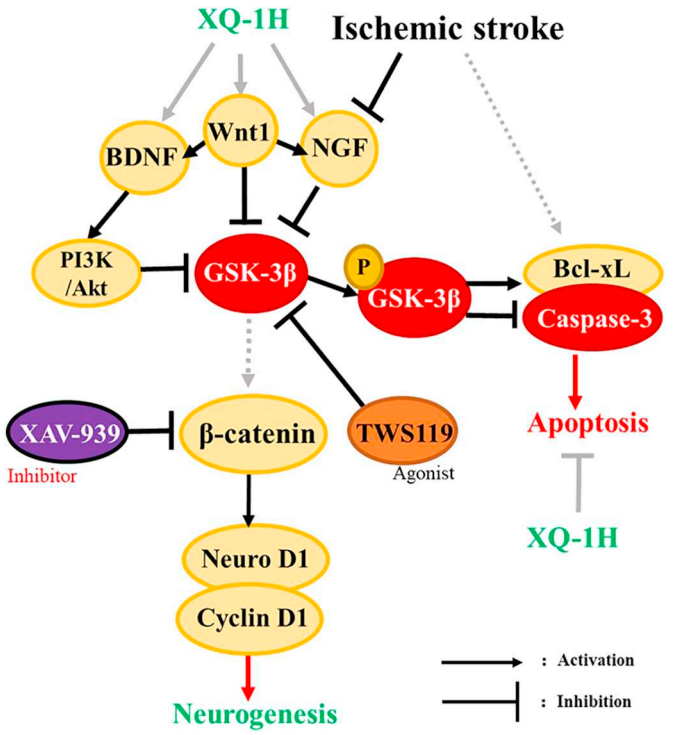

Fig. 7. A schematic illustration showing the key pathways regulated by XQ-1H, XAV-939 and TWS119 in cerebral ischemic injury. XQ-1H upregulated BDNF and NGF by causing activation of the PI3K/Akt pathway, which, in turn, induced Wnt/GSK-3β/β-catenin pathways for anti-apoptosis and neurogenesis related components, including Bcl-xL, Caspase-3, Neuro D1 and Cyclin D1; XAV-939 inhibited Wnt pathway through reinforcing the β-catenin destruction complex, while TWS119 indirectly induced Wnt pathway via GSK-3β inhibition.

5.7. Measurement of infarct size

Mice were sacrificed 1 h after drug administration on the third day and seventh day, and whole brain specimens were removed. The mouse brain was cut into five 2 mm-thick coronal slices and put into 1% of 2, 3, 5 - Triphenyltetrazolium chloride (TTC, Sangon biotechnology co., LTD., Shanghai, China; Cat# 2273GR005) at 37 °C for 15 min. The unstained area was the infarct area. Image J software (Version 1.4.3; National Institutes of Health, Bethesda, Maryland, USA) was adopted to analyze brain images to determine the infarct area as follows:

Infarct size (%) = (contralateral area-ipsilateral non-infarct area)/ contralateral area × 100%.

5.8. Evaluation of cerebral edema

Brain water content was used as the index to evaluate cerebral edema [51]. The brain was measured the wet weight before dried in the 110 °C overnight and weighed again for the dry weight. The brain water content was calculated as follows:

Brain water content (%) = ((wet tissue weight - dry tissue weight)/ wet tissue weight) × 100%.

5.9. Analysis of blood-brain barrier (BBB) integrity

The exudation rate of Evans blue (EB, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China; Cat# E-2129) was photographed and measured to evaluate the BBB permeability. After 70 h of MCAO, 2% EB solution (2 mL/kg) was injected into the femoral vein of mice. Two hours later, the left ventricle was perfusion with cold PBS to remove EB. The brain was removed and divided into left and right hemispheres and weighed separately before placed the formamide solution in 56 °C for 24 h. OD value of supernatant was determined at 632 nm after homogenization and centrifugation. EB tissue leakage was calculated as follows [52]:

EB leakage rate (%)
= (right hemisphere OD/right hemisphere weight)
/(left hemisphere OD/left hemisphere weight) × 100%。

5.10. Measurement of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) concentration

Mice were sacrificed 1 h after the third day of drug treatment, and ischemic penumbra was taken out to prepare tissue homogenate in ice water, which was centrifuged at 3000 rpm at 4 °C for 20 min, and the supernatant was collected for detection of BDNF (Excell Biotech Co., Ltd.; Taicang; China; Cat# EM025–96; Lot# 21H176) and NGF (Cusabio Biotech Co., Ltd., China; Cat# CSB-E04684m; Lot#T11032710) by ELISA kits according to instructions of the kits.

5.11. Brain histopathologic analysis

Hematoxylin and eosin (H & E) staining was used to detect the pathological changes after ischemic stroke. Seventy-two hours after stroke induction, the brains were prepared as previously described. The sections were cut into successive, paraffin-embedded coronal sections (6 μm) and stained with H & E staining. Histopathological changes induced by MCAO/R were recorded and photographed at a magnification of 200×. Pathological injury was scored on a scale of 0 to 4 according to a previous study with some modification: 0, normal; 1 focal loss of individual neurons; 2 multiple or large foci of neuronal loss along with reactive gliosis, involving one-half or less of the area of ipsilateral hemisphere; 3 obvious infarction, neuronal loss and reactive gliosis, involving greater than half of the cerebral hemisphere; 4 severe infarction, neuronal loss, more than half of the cerebral hemisphere [53,54].

5.12. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

1 h after the third day of drug administration, the apoptotic neurons after MCAO/R were detected by immunofluorescence assay and TUNEL staining as previously described [55]. The brain slides were incubated with anti-Neuronal Nuclei (NeuN) antibodies (1:500, Abcam, Cambridge, MA, USA Cat# ab177487) overnight at 4 °C. Subsequently, goat anti-rabbit IgG/Cy3 (1:200, Bioss, Beijing, China; Cat# bs-0295G-Cy3) was incubated for 2 h. The rinsed slides were stained using TUNEL kit (Keygenbiotech, Nanjing, China; Cat# KGA7071; Lot# 20181212) based on the manufacturer's instructions followed with DAPI staining for 30 min. Images were captured with a 10 × fluorescence microscope (LSM800, Carl Zeiss, Germany). Green TUNEL dots located in red neurons with a blue nucleus were identified as apoptotic neuron. The total number of neurons and the number of apoptotic neurons in each segment were counted and the ratio was calculated.

5.13. Immunofluorescence

Additional brain slides were blocked in 10% goat serum at room temperature for 2 h [56]. For BrdU, DNA was first denatured by incubating the tissue sections with 2 N HCl at 37 °C for 30 min [35] and HCl was neutralized in 0.1 M borate buffer. Then the following primary antibodies were used: rabbit monoclonal anti-β-catenin (1:50; Proteintech, Wuhan, China; Cat# 51067–2-AP), monoclonal mouse anti-BrdU antibody (1:400; Cell Signaling Technology Inc., MA, USA; Cat# 5292), rabbit monoclonal anti-doublecortin (DCX, 1:200; Cell Signaling Technology Inc., MA, USA; Cat# 4604) and monoclonal rabbit anti-

NeuN antibody (1:500, Abcam, Cambridge, MA, USA Cat# ab177487). Sections were incubated overnight at 4 °C with primary antibodies and thereafter incubated with secondary antibodies, which were goat anti-mouse IgG/FITC antibody (1:200; Bioss, Beijing, China; Cat# bs-0296G-FITC) and/or goat anti-rabbit IgG/Cy3 antibody (1:200; Bioss, Beijing, China; Cat# bs-0295G-Cy3). Then slides were stained with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime Institute of Biotechnology, Shanghai; Cat# C1005). The neurogenesis after ischemia was analyzed in three regions of interests, located in SGZ, SVZ and peri-infarct cortex, respectively. The analysis included the quantification of the percentage of $BrdU^+$ cells coexpressing the mature neuronal marker NeuN, as well as the percentage of DCX^+ immature neurons coexpressing the DAPI staining cells. Images were taken using a fluorescence microscope (LSM800, Carl Zeiss, Germany) through 20 or 10 × objective lens.

5.14. Real-time PCR

Changes in mRNA levels of Wnt1, β-catenin and GSK-3β were detected in brain tissues, and real-time PCR was performed as described previously [57]. Total RNA from ischemic brain tissue was extracted using Trizol reagent (Vazyme Biotech Co., Ltd., Nanjing, China). Then a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech Co., Ltd., Nanjing, China) was used to reverse-transcribe the total RNA into cDNA according to the manufacturer's instructions. Real-time PCR was performed using quantitative PCR (Mastercycler ep realplex, Eppendorf) in the presence of fluorescent dyes (SYBR Green I). The comparative threshold cycle (CT) value method was used to determine the quantities of expression and eq. $2^{-\Delta\Delta\ CT}$ was to determine the fold changes. The mRNA levels were normalized to β-actin of the same sample and were displayed as the fold changes vs. sham group. The primers (Sangon Biotech Co., Ltd., Shanghai, China) were as follows:

Wnt1 F: ACAGCGTTCATCTTCGCAATCACC; R: AAATCGATGTTGT CACTGCAGCCC; β-catenin F: TCAGCTCGTGTCCTGTGAA; R: CAGGTC AGCTTGAGTAGCCATT; GSK-3β F: AGTGGTGAGAAGAAAGATGAGGT; R: AAGAGTGCAGGTGTGTCTCG; β-actin F: TGTTACCAACTGGGACG AGA; R: CTTTTCACGGTTGGCCTTAG.

5.15. Western blotting analysis

The brain tissues of different batches of mice were harvested to extract total protein, cytoplasmic/nucleoprotein and phosphorylated protein according to the instructions of the respective extraction kits (Beyotime Institute of Biotechnology, Shanghai, China). Protein content was estimated by a BCA kit (Beyotime Institute of Biotechnology, Shanghai, China; Cat# P0012S). Equivalent protein samples (50 μg) were separated by 10% SDS/PAGE gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane (Biosharp, Hefei, China). The membrane was incubated in 5% fat-free milk in TBST (0.01 M Tris, 0.5% Tween-20). Then, the membranes were incubated with following antibodies: anti-rabbit polyclonal antibody Wnt1 (1:1000, Affinity Bioscience, USA; Cat#AF5315), BDNF (1:500; Affinity Bioscience, USA; Cat# DF6387); NGF (1:500; Affinity Bioscience, USA; Cat# DF6061); Neuro D1 (1:50; Wanleibio, Shenyang, China; Cat# WL02498); Cyclin D1(1:50; Wanleibio, Shenyang, China; Cat# WL01435a); β-catenin (1:1000; Proteintech, Wuhan, China; Cat# 51067–2-AP), GSK-3β (1:1000; Proteintech, Wuhan, China; Cat# 22104–1-AP); p-GSK-3β (Phospho-Ser9, 1:1000; Zen-bio; Chengdu, China; Cat# 310010); Akt (1:500; Wanleibio, Shenyang, China; Cat# WL0003b); p-Akt (Phospho-Ser473; Wanleibio, Shenyang, China; Cat# WLP001); Caspase 3 (1:500; Wanleibio, Shenyang, China; Cat# WL01927); Cleaved-caspase 3 (1:500; Wanleibio, Shenyang, China Cat# WL03707); Bcl-xL (B-cell lymphoma-extra-large, 1:500; Wanleibio, Shenyang, China; Cat# WL01776), mouse polyclonal antibody GAPDH (1:1000; Abclonal, Nanjing, China; Cat# AC002), β-acin (1:1000; Beyotime biotechnology research institute, Shanghai, China; Cat# AF0003) or rabbit polyclonal antibody Lamin B1 (Abways technology, Shanghai, China; Cat# AB0054) at 4 °C overnight. The next day, the PVDF membrane was incubated with goat anti-rabbit secondary antibody (1:1000; Beyotime Institute of Biotechnology, Shanghai, China) or sheep anti-mouse secondary antibody (1:1000; Beyotime Institute of Biotechnology, Shanghai, China) and visualized via chemiluminescence. The results were normalized to β-actin, GAPDH or Lamin B1 and quantified with Gel-Pro analyzer 4 (JS-860B, Shanghai Peiqing Science & Technology Co., Ltd.).

5.16. Statistical analysis

The data were expressed as mean ± standard error of mean (S.E.M). IBM SPSS 20.0 software was used for statistical analysis. Statistical differences between groups were analyzed using two-way ANOVA followed by Bonferroni test to evaluate the dependency of the independent variables XQ-1H, XAV-939 and TWS119. The neurological scores, corner turn test and pathological scores of mice in administration groups were compared with MCAO/R group using the Kruskal-Wallis test. Mann-Whitney *U* test was used for real-time PCR comparison between groups. $p < 0.05$ was considered statistically significant. Photoshop CS6, Gel-pro analyzer 4, GraphPad Prism Version 7.0 and Zen blue 2.1 (Zeiss) were used for statistical analysis and cell counting.

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Author contributions

D.X. performed most of the experiments and was involved in writing a draft manuscript; K.H. and F.L. conducted some of the experiments and performed data analysis. Y.L and W.F. were involved in experimental design; S.C. was involved in writing and modification of the final manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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